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(54) Title: IMMUNOGLOBIN-LIKE DOMAINS WITH INCREASED HALF LIVES

(57) Abstract

Disclosed are recombinant vectors encoding immunoglobulin-like domains and portions thereof, such as antibody Fc-hinge fragments, subfragments and mutant domains with extended biological half lives. Methods of producing large quantities of such domains, heterodimers, and fusion proteins following expression by host cells are also reported. Described are antibody Fc and Fc-hinge domains, which have the same *in vivo* stability as intact antibodies; and domains engineered to have increased *in vivo* half lives. These DNA constructs and protein domains will be useful as templates for *in vitro* mutagenesis and high resolution structural studies; for immunization and vaccination; and for the production of recombinant antibodies or chimeric proteins with increased stability and longevity for therapeutic and diagnostic uses.

INTERNATIONAL SEARCH REPORT

International application No.
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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 23,32
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 23 and 32 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 97/03321

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 C07K16/00 A61K47/48 G01N33/557 C07K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 26, no. 3, March 1996, WEINHEIM, GERMANY, pages 690-696, XP000676642 V. GHETIE ET AL.: "Abnormally short serum half-lives of IgG in beta2-microglobulin-deficient mice." cited in the application see abstract --- -/--	1-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR IMMUNOLOGY, vol. 29, no. 2, February 1992, OXFORD, GB, pages 213-220, XP000676636 E. WAWRZYNCZAK ET AL.: "Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side chains of the CH2 domains." see abstract ---	1-33
A	MOLECULAR IMMUNOLOGY, vol. 30, no. 4, March 1993, OXFORD, GB, pages 379-386, XP000676597 J. BATRA ET AL.: "Insertion of constant region domains of human IgG1 into CD4-PE40 increases its plasma half-life." see abstract ---	1-33
A	WO 94 04689 A (THE GOVERNMENT OF THE USA) 3 March 1994 see the whole document ---	1-33
A	EP 0 327 378 A (THE TRUSTEES OF COLUMBIA UNIVERSITY) 9 August 1989 see page 5, line 5 - line 12 ---	1-33
A	WO 93 22332 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 11 November 1993 see examples 5-7 see claims -----	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/03321

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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DESCRIPTION

IMMUNOGLOBIN-LIKE DOMAINS WITH INCREASED HALF LIVES

1. FIELD OF THE INVENTION

The present invention relates generally to the field of the transport of serum proteins and antibodies mediated by the Fc receptor, FcRn, and further to the effect on serum half life of agents that interact with the Fc receptor in a pH dependent way.

2. DESCRIPTION OF RELATED ART

IgGs constitute the most prevalent immunoglobin class in the serum of man and other mammals and are maintained at remarkably constant levels. Recent studies indicate that the major histocompatibility complex (MHC)-class I related receptor, FcRn, is involved in the homeostasis of serum IgGs (Ghetie *et al.*, 1996; Junghans and Anderson, 1996; Israel *et al.*, 1996). This receptor most likely acts as a salvage receptor, and this would be consistent with its known ability to transcytose IgGs in intact form across the neonatal gut (Wallace and Rees, 1980; Rodewald and Kraehenbuhl, 1984) and yolk sac (Roberts *et al.*, 1990; Israel *et al.*, 1995) or placenta (Kristoffersen and Matre, 1996; Simister *et al.*, 1996; Leach *et al.*, 1996). The interaction site of FcRn on mouse IgG1 (mIgG1) has been mapped using site-directed mutagenesis of recombinant Fc-hinge fragments, followed by analysis of these fragments both *in vivo* and *in vitro*(Kim *et al.*, 1994b; Medesan *et al.*, 1996; 1997). From these studies, I253 (EU numbering (Edelman *et al.*, 1969)), H310, H435 and to a lesser extent, H436 play a central role in this interaction. These amino acids are located at the CH2-CH3 domain interface (Deisenhofer, 1981), and the mapping of the functional site to these residues is consistent with the X-ray crystallographic structure of rat FcRn complexed with rat Fc (Burmeister *et al.*, 1994b).

The FcRn interaction site encompasses three spatially close loops comprised of sequences that are distal in the primary amino acid sequence. The central role of Fc histidines in building this site accounts for the marked pH dependence (binding at pH 6.0, release at pH

7.4) of the Fc-FcRn interaction (Rodewald and Kraehenbuhl, 1984 ; Raghavan *et al.*, 1995; Popov *et al.*, 1996), as the pKa of one of the imidazole protons lies in this pH range. I253, H310, H435 and to a lesser degree, H436, are highly conserved in IgGs of both human and rodent IgGs (Kabat *et al.*, 1991). This, taken together with the isolation of a human homolog of FcRn (Story *et al.*, 1994), indicate that the molecular mechanisms involved in IgG homeostasis are common to both mouse and man and this has implications for the modulation of the pharmacokinetics of IgGs for use in therapy.

To date, in studies to identify the FcRn interaction site on Fc, mutations of Fc-hinge fragments have been made that reduce the serum half lives of the corresponding Fc-hinge fragments (Medesan *et al.*, 1997; Kim *et al.*, 1994a). The correlation between serum half life and binding affinity for FcRn is excellent for these mutated Fc-hinge fragments (Kim *et al.*, 1994b; Popov *et al.*, 1996), suggesting that if the affinity of the FcRn-Fc interaction could be increased, whilst still retaining pH dependence, this would result in an Fc fragment with prolonged serum persistence. Production of such a fragment would be a significant advance in the engineering of a new generation of therapeutic IgGs with improved pharmacokinetics such as increased persistence in the circulation. But to date, no such fragments have been produced.

Immunoglobulin Fc domains are also of great interest for purposes of studying the mechanisms of antibody stabilization, catabolism and antibody interactions with further molecules of the immune system. These include, depending on the class of antibody, interactions with complement, and binding to specific receptors on other cells, including macrophages, neutrophils and mast cells. More detailed knowledge of the biology of Fc regions is important in understanding various molecular processes of the immune system, such as phagocytosis, antibody-dependent cell-mediated cytotoxicity and allergic reactions.

The production of a longer-lived Fc fragment that has increased binding to FcRn would be attractive, since such a fragment could be used to tag therapeutic reagents. Chimeric proteins produced in this manner would have the advantage of high *in vivo* stability which would allow fewer doses of the agent to be used in therapy and possibly even allow lower doses of the agent to be used through its increased persistence in the bloodstream.

Unfortunately, methodology for generating proteins, such as antibody fragments, with increased serum persistence has not yet been developed.

SUMMARY OF THE INVENTION

The present invention seeks to overcome deficiencies in the art by providing functional proteins, antibodies or other agents that have an increased serum half-life through the interaction with Fc receptor (FcRn). These functional agents include any molecule that binds to FcRn in a pH dependent way such that binding affinity is strong at about pH 6 to about pH 6.5 relative to binding at pH 7.4. Physiologically, this allows the agent to be salvaged by FcRn at lower pH and released into the essentially neutral pH environment of the serum. The present disclosure includes protein and peptide compositions having altered serum half-lives relative to IgG, methods of making such proteins or peptides, either starting with a known sequence or by screening random sequences, and methods of screening unknown candidate agents for pH dependent FcRn binding. In addition, disclosed herein are methods of making an agent with altered serum half-life by conjugating or otherwise binding of that agent to a moiety identified as having an increased serum half-life through its interaction with FcRn. Such agents would include, but are not limited to antibodies, fragments of antibodies, hormones, receptor ligands, immunotoxins, therapeutic drugs of any kind, T-cell receptor binding antigens and any other agent that may be bound to the increased serum half life moieties of the present invention.

Also disclosed are methods of increasing the FcRn binding affinity of an FcRn binding protein or peptide so that the protein or peptide will have an increased serum half-life. These methods include identifying amino acids that directly interact with FcRn. These amino acids may be identified by their being highly conserved over a range of species, or by any other method. Other methods would include, for example, mutation or blocking of the amino acid and screening for reduced binding to FcRn, or by a study of three dimensional structure of the interaction, or by other methods known in the art. When those residues are identified that directly interact, then secondary amino acids are identified whose side chains are in the spatial vicinity of the direct interaction. In the case of antibodies, these secondary amino acids often occur in loops so that they are exposed to the solvent. In this way, mutation of these amino

acids is not expected to disrupt the native protein structure. These identified secondary amino acids are then randomly mutated and the mutated proteins or peptides are then screened for increased binding affinity for FcRn at about pH 6 relative to the non-mutated protein or peptide. This method is applicable to any protein or peptide that binds FcRn in a pH dependent way and all such proteins or peptides would be encompassed by the present claimed invention. It is also understood that random mutation, in and of itself, does not constitute the invention, and that the secondary amino acids may be specifically mutated or modified or derivatized in any way known in the art and then screened for the effect on FcRn binding.

In certain broad aspects, the invention encompasses the design and production of recombinant antibody or antibody Fc-hinge domains engineered to have increased *in vivo*, or serum half lives. The Fc-hinge domain mutants with increased serum half lives of the present invention are generally defined as mutants in which one or more of the natural residues at the CH2-CH3 domain interface of the Fc-hinge fragment have been exchanged for alternate amino acids. Such Fc-hinge domain mutants may also be functionally defined as mutants which exhibit impaired SpA (Staphylococcal protein A) binding. In preferred embodiments, the increased half-life Fc-hinge mutants will have changes in certain amino acids between about residue 252 and about residue 436, which have been discovered to form, or be in close proximity to, the 'catabolic control site'.

In a further embodiment, the invention encompasses the isolation of peptides or agents that bind to FcRn with an affinity that may not necessarily be greater than that of the IgG:FcRn interaction yet the peptides or agents still have a measurably longer half life than a similar peptides or agents that do not bind to FcRn in a pH dependent manner as described herein. It is envisioned that such peptides or agents are useful as a stabilization "tag" for a therapeutic agent or protein.

More particularly, the present invention concerns mutant Ig domains and antibodies containing domains in which one or more of the following amino acids have been exchanged for other residues: threonine (thr) at position 252, threonine at position 254, threonine at position 256 (wherein the amino acids are numbered according to Kabat *et al.*, (1991)). To increase the half life of an Fc-hinge domain, or intact antibody, any of the above residues may

be substituted for any other amino acid residue and then variants that have higher affinity for FcRn may be selected using bacteriophage display, for example, or by any other method known to those of skill in the art. Substitution can advantageously be achieved by any of the molecular biological techniques known to those of skill in the art, as exemplified herein below, or even by chemical modification.

Certain increased half-life antibodies or domains will be those which include one or more of the following substitutions on the Kabat numbering system, or their equivalents on different numbering systems: threonine (thr) 252 to leucine (leu) 252, threonine 254 to serine (ser) 254, threonine 256 to phenylalanine (phe) 256. An example as disclosed herein is the triple mutant termed LSF which contains the three mutations: threonine 252 to leucine 252, threonine 254 to serine 254, threonine 256 to phenylalanine 256.

The production of Fc-hinge domains with longer *in vivo* half lives is an advantageous development in that it further delineates the site for the control of IgG1 catabolism to a specific region of the Fc-hinge fragment, and in practical terms, it has several important applications. It allows the design and construction of antibody molecules, domains, or fragments, such as bivalent Fab fragments, with longer half lives. These would be generally useful in that the slower biological clearance times would result in fewer administrations of any antibody or vaccine such that fewer "booster" vaccinations may be required. Furthermore, these molecules with longer half lives can be used to tag other therapeutic molecules, such as vaccine molecules. The catabolic site delineated in this invention is distinct from the ADCC and complement fixing sites. This is important as antibodies may be produced which are completely functional and which have longer half lives. Other important uses include, for example, antibody-based systemic drug delivery, the creation of immunotoxins with longer lives or even antibody-based immunotherapy for chronic illnesses or conditions such as hay fever or other allergic reactions, or treatment of T-cell mediated autoimmune disorders by anti-T-cell receptor antibodies or T-cell antigens.

The Fc-hinge domain mutants may also be employed in embodiments other than those involving clinical administration, for example, in the isolation of receptors involved in IgG

catabolism. To this end, one may use screening assays or differential screening assays in which the mutants would exhibit binding or increased binding to a potential catabolic receptor.

The discoveries disclosed herein concerning antibody catabolism are also envisioned to be useful to increase the *in vivo* half life of virtually any recombinant protein, and particularly a recombinant antibody, which one desires to administer to a human or animal. An antibody or recombinant protein that was found to be cleared from the body more quickly than ideally desired could be engineered at the residues identified herein, or in the vicinity of amino acids that are discovered to directly interact with FcRn, such that its *in vivo* half life was increased.

In certain other embodiments, the present invention contemplates the creation of recombinant molecules, particularly antibody constructs, including vaccines and immunotoxins, with increased *in vivo* half lives. Longevity of recombinant molecules is often needed, and several protocols would benefit from the design of a molecule which would be more slowly removed from circulation after exerting its designed action. This may include, for example, antibodies administered for the purpose of scavenging pathogens, toxins or substances causing biological imbalances and thereby preventing them from harming the body; and antibodies designed to provide long-term, systemic delivery of immunotherapeutic drugs and vaccines.

To generate a domain, antibody or antibody construct with a longer half-life, one would modify the natural residues at the CH2-CH3 domain interface of the Fc-hinge which either form the "catabolic control site" or are in close proximity to it. Several such catabolism controlling mutations are described herein which may be straightforwardly engineered into an antibody molecule or antibody conjugate. These include, substituting another residue for threonine 252, threonine 254, threonine 256, methionine 309, glutamine 311 and/or asparagine 315 (Kabat *et al.*, 1991). The present invention also provides an advantageous method for determining other residues important for catabolism control.

The proteins or peptides of the present invention may be expressed from recombinant plasmids or expression vectors adapted for expression of immunoglobulin-like domains, such as antibody domains, or other proteins or peptides in recombinant host cells. Recombinant plasmids thus may comprise a DNA segment coding for one or more immunoglobulin-like

domains. Accordingly, any one or more of a wide variety of immunoglobulin-like domains or other protein or peptide may be incorporated into a recombinant vector and expressed in a host cell in accordance herewith. These include, but are not limited to, variable or constant domains from IgG, IgM, IgA, IgD, IgE, T cell receptors, MHC class I or MHC class II, and also, CD2, CD4, CD8, CD3 polypeptides, Thy-1 and domains from the PDGF receptor, N-CAM or Ng-CAM.

In certain embodiments, the present invention concerns the expression and production of antibody constant domains. The production of antibody Fc-hinge, Fc, CH2-hinge or CH3 domains is preferred, with Fc-hinge or Fc domains being particularly preferred due to their longer *in vivo* half lives. In other instances, the production of Fc-hinge domains (or antibodies incorporating such domains) with mutations at thr 252, thr 254 or thr 256 is preferred as these have specifically longer half lives. Such mutants are exemplified by thr 252 to leu 252, thr 254 to ser 254 and thr 256 to phe 256.

Various segments or subfragments of any of the above domains, as well as other variable or constant domains, may also be employed in accordance herewith. These domains include, for example, the immunoglobulin domains CH1. Variations of immunoglobulin domains other than those specifically described above also fall within the scope of the invention. Such variations may arise from naturally-occurring or genetically engineered mutations, such as point mutations, deletions and other alterations affecting one or more amino acids or the addition of amino acids at the N or C termini.

Furthermore, while the invention has been illustrated with murine FcRn and immunoglobulin fragments, similar strategies are applicable to immunoglobulin-like domains or other proteins or peptides from a variety of other species, including mammals such as rat, and more particularly, human immunoglobulin-like molecules. In light of the structural similarity of the immunoglobulin-like domains, and the conservation of the immunoglobulin superfamily throughout evolution, it is contemplated that the techniques of the present invention will be directly applicable to the expression and recombinant production of an immunoglobulin-like domain from any given species.

Other DNA segments may also be included linked to the immunoglobulin-like domains described. For example, one or more recombinant antibody variable domains of varying specificities may be linked to one or more antibody constant domains, immunoglobulin constant domains, or even other proteins, such as bacteriophage coat protein genes, hormones or antigens, including T-cell receptor antigens. The antibody constant domains of the present invention may also be combined with another immunoglobulin domain, or indeed, with any other protein. The immunoglobulin constant domains may be variously expressed as a single domain, such as a CH3 domain; or in combination with one, two, three or more domains, such as, for example, as a CH2-hinge domain, an Fc domain, or an entire Fc-hinge domain. In particular embodiments, discussed in more detail below, Fc or Fc-hinge domains may be linked to any protein to produce a recombinant fusion with enhanced biological stability, or certain mutants may be employed to create antibodies or fusion proteins with increased half lives.

Once expressed, any of the products herein could be radiolabeled or fluorescently labeled, or attached to solid supports, including sepharose or magnetic beads or synthetic bilayers such as liposomes. The products could also be linked to carrier proteins such as bovine serum albumin. The Fc constant domains, or constant domains in combination with other proteins, could also be linked synthetically to co-receptors such as the extracellular domains of CD4 or CD8.

Recombinant, or cloning, vectors are included in one aspect of the present invention. Such vectors and DNA constructs will be useful not only for directing protein expression, but also as for use as templates for *in vitro* mutagenesis. Vectors will generally include a leader sequence, preferably *pel*B (Better *et al.*, 1988), although other leader sequences may be used, for example, alkaline phosphatase (phoA) or *omp*A. In a preferred embodiment, the *pel*B leader segment is modified with a unique restriction site, such as *Nco*I, allowing insertion of antibody variable domain genes. Introduction of such restriction sites is a convenient means of cloning in a DNA segment in the same reading frame as the leader sequence.

Modification of the leader sequence DNA may be achieved by altering one or more nucleotides employing site-directed mutagenesis. In general, the technique of site specific mutagenesis is well known in the art as exemplified by publications (Carter *et al.*, 1985;

Sambrook *et al.*, 1989). As will be appreciated, the technique typically employs a phagemid vector which exists in both a single stranded and double stranded form. Alternatively, mutants may be generated by using the PCR ™. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing *et al.*, 1981) or pUC 119. These vectors are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, methods of site-directed mutagenesis employing double stranded plasmids or phagemids and the like are also well known in the art and may also be used in the practice of the present invention.

Site directed mutagenesis in accordance herewith is performed by first obtaining a single stranded vector which includes within its sequence the DNA sequence encoding a leader sequence, *pel*B being used herewith. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Narang *et al.*, (1980). The primer is annealed with the single stranded vector and subjected to DNA polymerizing enzymes such as the *E. coli* polymerase I Klenow fragment. In order to complete the synthesis of the mutation bearing strand, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. The heteroduplex may be transformed into a bacterial cell, with *E. coli*. being preferred. Clones are screened using colony hybridization and radiolabeled mutagenic oligonucleotides to identify colonies which contain the mutated plasmid DNA (Carter *et al.*, 1985). PCR™ directed mutagenesis, using double-stranded DNA templates, is particularly suitable for generating increased half life mutants. PCR ™ mutagenesis typically involves the use of a primer encoding one or more alternate or random amino acid in one or more amplification reactions.

Constructs may also include a "tag" useful for isolation and purification of the expressed polypeptide product. Tags are relatively short DNA segments fused in-frame with a sequence encoding a desired polypeptide, such as polyhistidine, which have the function of facilitating detection, isolation and purification. For example, affinity peptides may be encoded by the segments, allowing isolation by selective binding to specific antibodies or affinity resins. Any of a number of tags may be used, including the *c-myc* tag, $(his)_6$ tag, decapeptide tag

(Huse *et al.*, 1989), Flag™ (Immunex) tags and so forth. A number of the tags are also useful for the detection of expressed protein using Western blotting (Ward *et al.*, 1989; Towbin *et al.*, 1979).

(*His*)$_6$ tags, for example, are preferable for purifying secreted polypeptide products on affinity metal chromatography columns based on metals such as Ni^{2+}. The (*his*)$_6$ peptide chelates Ni^{2+} ions with high affinity. Polypeptide products containing these residues at the N or C termini bind to the affinity columns, allowing polypeptide impurities and other contaminants to be washed away as part of the purification process. Polypeptide products can then be eluted from the column with high efficiency using, for example, 250 mM imidazole.

Peptide tags, or linkers, may also be incorporated into the immunoglobin product. For single chain Fv or T cell receptor (TCR) fragments, preferred linker peptides include a 15-mer, for example, (gly$_4$ser)$_3$, or other linkers, such as those described in Filpula and Whitlow (1991).

As mentioned above, recombinant vectors of the present invention may also include DNA segments encoding various other proteins. In particular, it is envisioned that recombinant vectors encoding antibody Fc-hinge or Fc domains may also include DNA segments encoding other proteins, or fragments thereof, particularly where one wishes to produce the protein in a form that has a longer serum half life. It is envisioned that the serum stability of proteins or peptides intended for administration to animals or humans may be increased in this manner. Examples of such proteins or peptides include, for example, interleukin-2, interleukin-4, γ-interferon, insulin, T cell epitopes and the like, and even TCR V_α, V_β. A variety of synthetic drugs could, likewise, be stabilized in this manner.

DNA segments encoding such proteins may be operatively incorporated into a recombinant vector, in frame with the Fc-based domain, whether upstream or downstream, in a position so as to render the vector capable of expressing a protein:Fc domain fusion protein (or a protein:Fc-hinge domain fusion protein). Techniques for the manipulation of DNA segments in this manner, for example, by genetic engineering using restriction endonucleases, will be

known to those of skill in the art in light of both the present disclosure and references such as Sambrook *et al.* (1989).

The invention has been illustrated with prokaryotic host cells, but this is not meant to be a limitation. The prokaryotic specific promoter and leader sequences described herein may be easily replaced with eukaryotic counterparts. It is recognized that transformation of host cells with DNA segments encoding any of a number of immunoglobulin-like domains will provide a convenient means of producing fully functional proteins, such as for example, functional IgGs. Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will, of course, process the genomic transcripts to yield functional mRNA for translation into protein. Increased half life mutant domains and antibodies may be produced in glycosylated form in eukaryotic systems which fix complement, and mediate ADCC.

It is similarly believed that almost any eukaryotic expression system may be utilized for the expression of proteins and peptides of the present invention, *e.g.*, baculovirus-based, COS cell-based, myeloma cell-based systems could be employed. Plasmid vectors would incorporate an origin of replication and an efficient eukaryotic promoter, as exemplified by the eukaryotic vectors of the pCMV series, such as pCMV5.

For expression in this manner, one would position the coding sequences adjacent to and under the control of the promoter. It is understood in the art that to bring a coding sequence under the control of such a promoter, one positions the 5′ end of the translation initiation site of the translation reading frame of the protein between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3′ of) the chosen promoter.

Where eukaryotic expression is contemplated, one will also typically desire to incorporate into the transcriptional unit, an appropriate polyadenylation site (*e.g.*, 5′-AATAAA-3′) if one was not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

As used herein the term "engineered" or "recombinant" cell is intended to refer to a cell into which a recombinant gene, such as a gene encoding an immunoglobulin-like domain, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinant gene that is introduced by transfection or transformation techniques. Engineered cells are thus cells having a gene or genes introduced through the hand of man.

Suitable host cells useful in the practice of the invention include gram-negative organisms and might include *Serratia marcescens, Salmonella typhimurium* and similar species. A particularly preferred host cell is *Escherichia coli* and the several variants of *E. coli* that are readily available and well known to those of skill in the art.

A particular aspect of the invention is a method for the production of immunoglobulin-like domains, such as, native or mutant antibody constant domains, or subfragments or fusion proteins thereof. To produce such domains or modified domains, a gram-negative microorganism host cell is first transformed with any of the disclosed recombinant vectors, and then cultured in an appropriate bacterial culture medium under conditions to allow expression of the immunoglobulin-like domain(s), which may be subsequently isolated.

Culturing typically comprises growing and induction. Growing is conveniently performed in such media as Luria broth plus 1% glucose, 4 x TY (double strength 2 x TY) plus 1% glucose, minimal media plus casamino acids and 5% w/v glycerol with temperatures in the range of 20°C to about 37°C, preferably between 25-30°C. In preferred embodiments, the media will contain a selection agent, such as ampicillin at a concentration of 0.1 mg/ml to select bacterial cells which contain the expression plasmid. Naturally, one will choose a particular selection agent in conjunction with the plasmid construct originally employed, as is known to those of skill in the art.

Induction of expression is typically performed at a point after growth has been initiated, usually after 12-16 hours at 30°C. This length of time results in the cells being in the early stationary phase at the induction stage. If the growth media contains glucose, the cells are pelleted and washed prior to addition of an inducer, such as isopropylthiogalactopyranoside

(IPTG) at a concentration of 0.1-1 mM, since glucose inhibits induction of expression. Again, a variety of other inducers may be employed, according to the vector construct originally used, as is known in the art. Cells may be grown for shorter periods prior to induction, for example for 6-10 hours, or to the mid-exponential stage of growth. Cells are induced for 5-28 hours. Five to six hours of induction is a preferred induction time if the protein is to be isolated from the periplasm, since longer induction times result in the protein leaking into the culture supernatant. However, it may be desirable to isolate product from the external medium, in which case one would prefer using longer induction times. Temperatures in the range of 20°C to 37°C may be used as growth and induction temperatures, with 25°C being a preferred induction temperature.

Isolating polypeptide products produced by the microbial host cell and located in the periplasmic space typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis, but preferably by osmotic shock. Once cells are disrupted, cells or cell debris may be conveniently removed by centrifugation or filtration, for example. The proteins may be further purified, for example, by affinity metallic resin chromatography when appropriate peptide tags are attached to the polypeptide products.

Alternatively, if the induction period is longer than 8 hours (at 25°C, for example), so that the protein leaks into the culture supernatant, cells may be removed from the culture by centrifugation and the culture supernatant filtered and concentrated (for example, 10-20 fold). Concentrated supernatant is then dialyzed against phosphate buffered saline and separation achieved by column chromatography, such as affinity or adsorption chromatography. An example is separation through Ni^{2+}-NTA-agarose to separate appropriately tagged proteins such as those carrying a $(his)_6$ tag. When these tags are used in the construction of an expression vector, histidine tags are particularly preferred as they facilitate isolation and purification on metallic resins such as Ni^{+2}- NTA agarose.

As used herein, the term "biologically stable protein" is intended to refer to a protein which has been modified resulting in increased serum half life with respect to the original protein. This term encompasses both known recombinant proteins and also proteins for which the recombinant form has not yet been reported. As such, increased biological stability may be

6 than at pH 7.4 and comparing the binding affinity of said selected agent to FcRn to the binding affinity of IgG to FcRn under identical conditions, wherein an increased binding affinity for FcRn relative to the binding affinity of IgG is indicative of an agent with an increased serum half-life. Certain preferred candidate agents may be a peptide or polypeptide, or even an antibody or a fragment of an antibody. In alternate embodiments the peptide may be selected from a random peptide library, or may be a randomly mutated protein, or even a synthetic peptide.

In certain embodiments, the invention may also be a method of increasing the serum half-life of a therapeutic agent comprising conjugating said therapeutic agent to an agent having an increased serum half-life relative to the serum half-life of IgG identified by the methods disclosed and claimed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic representation of portions of the plasmids used for the expression and secretion of immunoglobulin constant region fragments in *E. coli*. a) CH3 domain; b) CH2-hinge; c) Fc fragment and d) Fc-hinge fragments. The lacz promoter is represented by open circles, the pelB leader by hatched boxes, the immunoglobulin domains [hinge region (H) and CH2, CH3 domains] by open boxes and the his_6 peptide tag (his) by filled-in boxes.

FIG. 2A. Clearance curves for recombinant $[CH2\text{-}hinge]_2$. The curves are biphasic with a rapid α phase (representing equilibration of the injected protein between the intra- and extra vascular space; the α phase of the proteins are in part determined by the size) and a longer β phase (representing the catabolism of the protein in the intra-vascular space). The half life of the β phases of the fragments are given in Table I and these represent the biological half-lives of the proteins.

FIG. 2B. Clearance curves for glycosylated IgG1 molecule.

FIG. 3A. Binding of ^{125}I-labeled WT Fc-hinge and HQ-310/HN-433 mutant to SVEC cells. Open bars represent amount bound to cells following washes, and filled-in bars represent amount extracted from cell pellet following extraction with 2.5 mg/ml CHAPS.

FIG. 3B. Data from repeat of study shown in FIG. 3A.

FIG. 4A. Catabolism of ^{125}I-labeled mIgG1, Fc-hinge fragments and IgA. Closed triangle and + represent WT Fc-hinge; closed box and X represent HQ-310/HN-433 mutant in β2m+/+ (closed triangle and box) and β2m-/- (+ and X) mice.

FIG. 4B. Catabolism of ^{125}I-labeled mIgG1, Fc-hinge fragments and IgA. Open triangle and open box represent IgA; open diamond and X-ed box represents mIgG1 in β2m+/+ (open triangle and open box) and β2m-/- (open diamond and X-ed square) mice. For each protein, representative curves for one mouse from within each group are shown. These data are for mice of the C57BL/6 background.

FIG. 5. Clearance curves of Fc-hinge fragments in SWISS mice. Curves for one representative mouse from within each group are shown.

FIG. 6A. Regions of SPR sensorgrams showing dissociation of wild type (WT) (filled squares) and LSF mutant (filled triangles) Fc-hinge fragments at pH 6.0. Plots are drawn using BIAevaluation 2.1 software. Responses as a function of time are shown in response units (RUs).

FIG. 6B. Regions of SPR sensorgrams showing dissociation of WT (filled squares) and LSF mutant (filled triangles) Fc-hinge fragments at pH 7.4. The arrow indicates the point at which the buffer was changed from pH 6.0 to 7.4. Plots are drawn using BIAevaluation 2.1 software. Responses as a function of time are shown in response units (RUs). The bulk shift downwards due to the pH 7.4 buffer relative to the pH 6.0 buffer (latter used as a baseline) results in the negative RU values.

FIG. 7. Clearance curves of the murine IgG1 molecule and IgG1-derived fragments.

FIG. 8. Intestinal transfer of murine IgG1, Fab, Fc-papain and recombinant Fc fragments. The numbers of mice used for each study were 6(Fab), 12 (mIgG1), 16 (Fc-papain), 31 (WT Fc), 5 (HQ-310/HN-433), 5 (WT Fc in adult mice) and 14 (Fc-hybrid).

FIG. 9. Correlation between β phase half life and inhibition of transfer for the recombinant WT and mutant Fc fragments.

FIG. 10. Inhibition of binding of ^{125}I-IgG1 to isolated brush borders by unlabeled IgG1, WT and HQ-310/HN-433 mutant Fc fragments.

FIG. 11. Expression/phage display vector containing Fc gene (WT or mutant). Open circle = lacz promoter, hatched box = pelB leader, open box = WT Fc or HQ-310/HN-433 mutant, filled in box = c-myc tag and stippled box = cpIII gene. Single lines = backbone vector.

FIG 12A. Transcytosis of recombinant Fc-hinge fragments. The numbers in parentheses represent the number of mice used for each experiment. Maternofetal transmission of recombinant Fc-hinge fragments in SCID mice.

FIG 12B. Inhibition of intestinal transmission of radiolabeled mIgG1 by recombinant Fc-hinge fragments in BALB/c neonates. The value for H433A is not significantly different from that for WT Fc-hinge. (by Student's test, $p = 0.127$).

FIG. 13A. Binding of recombinant Fc-hinge fragments to FcRn. Percent inhibition of FcRn binding to mIgG1-Sepharose relative to binding in absence of inhibitor (average of three separate studies).

FIG. 13B. Binding of recombinant Fc-hinge fragments to SpA. Percentage of Fc-hinge fragment binding, to SpA-Sepharose relative to the binding of WT Fc-hinge (average of three separate studies).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention concerns the cloning and expression of immunoglobulin-like domains and engineered and mutant domains in host cells such that the immunoglobin-like product has increased serum persistence.

Disclosed herein are recombinant vectors encoding immunoglobulin-like domains and portions thereof, such as antibody Fc fragments and subfragments and Fc-hinge domains with extended *in vivo* half lives. Methods of producing large quantities of, for example, immunoglobulin Fc and Fc-hinge domains, which have the same *in vivo* stability as intact antibodies, are described, as are methods for producing antibodies and other molecules with increased half lives. These DNA constructs and protein domains are envisioned to be of various uses, such as in the production of immunotherapeutics or other stable recombinant proteins, or in the production of constructs.

As the invention is exemplified by the production of a variety of immunoglobulin-like domains, including antibody Fc-hinge, Fc, CH2-hinge and CH3 domains; and engineered Fc-hinge domains with extended *in vivo* half lives, such as, for example, the mutant termed LSF; it will be understood that other immunoglobulin-like domains may be expressed employing the methods of the present invention.

It is recognized that a considerable number of the key molecules of the immune system include homologous domains, the structure of which have been conserved throughout evolution. Such molecules are members of the immunoglobulin superfamily, which includes, not only antibodies and T cell receptors, but also MHC class I and II glycoproteins, the CD2, CD4 and CD8 cell-cell adhesion proteins, and various Fc receptors, all of which contain one or more immunoglobulin-like domains.

Each of these domains is typically about 100 amino acids in length and is thought to be folded into the characteristic sandwich-like structure made of two antiparallel β sheets, usually stabilized by a conserved disulfide bond. Many of these molecules are dimers or higher

oligomers in which immunoglobulin homology units of one chain interact with those in another.

Each immunoglobulin homology unit is usually encoded by a separate exon, and its seems likely that the entire supergene family evolved from a gene coding a single immunoglobulin homology unit similar to that encoding Thy-1 or β_2-microglobulin, which may have been involved in mediating early cell-cell interactions. Since a Thy-1-like molecule has been isolated from the brain of squids, it is probable that such a primordial gene arose before vertebrates diverged from their invertebrate ancestors some 400 million years ago. New family members presumably arose by exon and gene duplications, and similar duplication events probably gave rise to the multiple gene segments that encode antibodies and T cell receptors.

Apart from antibodies and the T cell receptor, among the best characterized proteins which contain immunoglobulin-like domains are the MHC molecules and the CD4 and CD8 glycoproteins. There are two main classes of MHC (major histocompatibility complex) molecules, class I and II, each consisting of a set of cell-surface glycoproteins. Both classes of MHC glycoproteins are heterodimers with homologous overall structures, the amino-terminal domains of which are thought to be specialized for binding antigen for presentation to T cells. But FcRn, an MHC class I homolog, has a distinct function *i.e.* the regulation of serum IgG levels.

Each class I MHC gene encodes a single transmembrane polypeptide chain, termed α chain, most of which is folded into three extracellular, globular domains. Each α chain is noncovalently associated with a nonglycosylated small protein, termed β_2-microglobulin. β_2-microglobulin and the α_3 domain, which are closest to the membrane, are both homologous to an immunoglobulin domain, and thus both proteins are members of the immunoglobulin superfamily. The two amino-terminal domains of the α chain, which are farthest from the membrane, contain the polymorphic (variable) residues that are recognized by T cells. T cells also recognize virally derived peptides bound to Class I molecules, and this is particularly important in cellular immunity.

In common with class I MHC molecules, class II MHC molecules are heterodimers with two conserved immunoglobulin like domains close to the membrane and two polymorphic (variable) amino-terminal domains farthest from the membrane. In these molecules, however, both chains span the membrane. There is strong evidence that the polymorphic regions of both classes of MHC molecules interact with foreign antigen and that it is the complex of MHC molecule and foreign antigen that is recognized by the T cell receptor.

CD4 and CD8 are expressed on the surface of helper and cytotoxic T cells, respectively. Both glycoproteins are thought to bind to invariant parts of MHC molecules, CD4 to class II and CD8 to class I MHC glycoproteins.

Other molecules have subsequently been shown to include immunoglobulin-like domains. These include, for example, the PDGF receptor, the extracellular domain of which is thought to be folded into five immunoglobulin-like domains. An increasing number of cell-surface glycoproteins that mediate cell-cell adhesion in vertebrates have also been identified as belonging to the immunoglobulin superfamily. These include N-CAM, a large, single-pass transmembrane glycoprotein which is expressed on the surface of nerve cells and glial cells and mediates Ca^{2+}-independent cell adhesion. The extracellular portion of the N-CAM polypeptide is also folded into five immunoglobulin-like domains. The L1 glycoprotein, also known as the neuron-glia cell-adhesion molecule, or *Ng-CAM*, is also a member of the immunoglobulin superfamily.

A: Isolation of FcRn Ligands that Have Increased Serum Persistaence

By randomly mutating regions that are in close proximity to the interaction site of FcRn on the Fc fragment, followed by selection for higher affinity binders from the library of mutants, variant Fc fragments with increased serum half lives can be isolated. These higher affinity mutants should still maintain their pH dependence of binding, as data indicate that this is an important facet of the way in which FcRn works, *i.e.*, binding to IgGs in an acidic compartment and then being released at about pH 7.4 into the serum. Thus, this method could be used to select any ligand (protein, peptide, or even non-protein ligand) that binds to FcRn in a pH dependent way, especially when FcRn is used in soluble form to isolate the ligand. In

fact, it is clear that using the disclosed invention one could isolate a ligand from either a library of synthetic chemical compounds, a peptide library or library of proteins with randomized surface loops, obtain the soluble protein or peptide in as little as one week by using standard isolation procedures well known to those of skill in the art, and then use these peptides (loops) or proteins to prepare synthetic ligands using the ACD database to identify homologs. Furthermore such FcRn ligands might be more useful than IgGs or fragments as they may well be smaller, and in the case of synthetic ligands, would be expected to be non-immunogenic. In this respect, the isolation of a ligand that has a lower affinity than IgG for FcRn, as well as those that have the same or higher affinities, is contemplated as being useful. For example, a ligand that has a several fold lower affinity than mIgG1 can still have a significantly longer half life than a similar ligand that has no detectable affinity for FcRn.

The uses of a molecule that could be used to increase the serum half life of drugs, proteins, peptides, *etc.* would be enormous. In principle such a molecule could be used to increase the serum persistence of any therapeutic reagent. Therefore the claimed invention is broadly applicable to an almost unlimited number of therapeutic uses for the treatment of diseases or disorders as it can be used to both reduce costs and discomfort to the patient by reducing the number of therapeutic doses are needed.

B: Antibody Constant Domains

The features of an immunoglobulin molecule that determine high stability *in vivo* were incompletely understood prior to the present invention. Previous studies indicate that the CH2 domain may play an important role in the control of catabolism of antibodies, and a recent study has also suggested that sequences in the CH3 domain may be involved (Ellerson *et al.*, 1976; Mueller *et al.*, 1990; Pollock *et al.*, 1990; Kim *et al.*, 1994a: Medesan *et al.*, 1997). The presence of carbohydrate residues on the CH2 domain appears to have a minor if significant effect on the stability, and the extent of the effect is dependent on the isotype (Tao and Morrison, 1989).

As part of the present work, recombinant CH2-hinge, CH3, Fc and Fc-hinge fragments derived from the murine IgG1 constant region have been expressed from host cells. The

fragments have been purified, radiolabeled and used in clearance studies in mice. The clearance rates have been compared with those of an Fv fragment and a complete glycosylated IgG1 molecule. The recombinant Fc-hinge fragments have stability properties that are very similar to those of the complete immunoglobulin molecule. In contrast, the monomeric CH2-hinge and CH3 fragments are both cleared rapidly and in a similar way to the Fv fragment. This indicates that sequences in both the CH2 and CH3 region are important for *in vivo* stability, and in addition, that glycosylation only plays a minor role in the control of the catabolism of this isotype.

The CH3 domain, Fc fragment and Fc-hinge fragment were all found to be homodimeric proteins. For the Fc and CH3 domain, the dimers are non-covalently linked, and are presumably stabilized by non-covalent interactions. For the Fc-hinge dimer, the fragments are covalently linked by -S-S- bridges between the hinge region cysteines.

A particularly important aspect of this study is the finding that the immunoglobulin Fc-hinge and Fc fragments, purified following expression in host cells, have the same *in vivo* stability as a native antibody molecule. This was determined by measuring the clearance rates of ^{125}I-radiolabeled immunoglobulin fragments *in vivo* as a function of time. Results from these studies demonstrated that the recombinant aglycosylated Fc-hinge or Fc fragments have similar stability *in vivo* as the complete glycosylated IgG1 molecule.

The recombinant aglycosylated Fc-hinge fragment was found to have a β phase similar to that of a complete glycosylated IgG1 immunoglobulin molecule. In fact the removal of Fc-hinge resulted in a slight decrease in half life (Kim *et al.*, 1995). These results indicate that for the murine IgG1 isotype the presence of carbohydrate residues does not appear to be necessary for *in vivo* stability, although it may still play a minor role. Previous data obtained using protein chemistry suggested that the CH2 domain is responsible for *in vivo* stability (Ellerson *et al.*, 1976) although a recent study indicated that residues in the CH3 domain may also be involved in the catabolism control of the murine IgG2a and IgG2b isotypes (Pollock *et al.*, 1990).

The present discoveries relating to stability are particularly important as the *in vivo* stability of aglycosylated Fc fragments has not been previously assessed (Nose *et al.*, 1990). Aglycosylated Fc fragments, in comparison with the glycosylated version (prepared by proteolysis of immunoglobulin produced by mammalian cells), are known to have reduced binding to complement C1q and greatly reduced binding to Fc receptors on monocytes (Nose *et al.*, 1990; Leatherbarrow *et al.*, 1985; Nose and Wigzell, 1983; Tao and Morrison, 1989). However, these advantageous properties would be of little significance if the aglycosylated molecules were found to be unstable. The inventors have been able to express aglycosylated Fc fragments which proved to be stable *in vivo*.

The production of the IgG1 Fc-hinge or Fc fragment in *E. coli* has allowed the important residues of this region involved in controlling antibody stability and catabolism *in vivo* to be elucidated. These results are described in Example 8. Furthermore, following the present invention, human Fc domains may now be produced in *E. coli*, allowing further detailed studies of the human protein. Additionally, the bacterial secretion of Fc or Fc-hinge domains, or Fc or Fc-hinge domain:fusion proteins, whether of murine or human origin, is envisioned to provide a convenient, economically attractive and rapid route for the production of novel proteins that have long serum persistence.

Following structural analyses, smaller regions of the Fc structure may be employed in protein chimeras, or fusion proteins, to produce biologically stable therapeutic agents. This is particularly useful for the production of therapeutic agents which cannot be obtained from other expression systems, such as mammalian cells, due to proteolysis. As such, the Fc-hinge or Fc domains of the present invention, or portions thereof, are proposed to be useful modules for both the tagging and stabilization of recombinant molecules, including chimeric proteins of therapeutic use.

C: Catabolic Site of the IgG Molecule.

Of the Ig class (IgA, IgE, IgM, IgD and IgG), the IgG molecule has the longest *in vivo* half life (Zuckier *et al.*, 1990). The region of the IgG molecule that controls catabolism (the 'catabolic site') has been known for several decades to reside in the Fc fragment. This work,

carried out initially by Spiegelberg and Weigle (1966) and later confirmed by many others (reviewed in Zuckier *et al.*, 1990), indicated that the Fc fragment produced by proteolysis has the same *in vivo* half life as the complete IgG molecule. Works by Dorrington and colleagues (Dorrington and Painter, 1974; Ellerson *et al.*, 1976; Yasmeen *et al.*, 1976) showed that a CH2 domain fragment produced by trypsin digestion had the same half life as that of the complete IgG molecule. Although both earlier and more recent data suggest that the CH2 domain is involved in the control of IgG catabolism, some of these data are not inconsistent with the additional involvement of the CH3 domain (Arend and Webster, 1977; Dima *et al.*, 1983; Mueller *et al.*, 1990; Kim *et al.*, 1994a; Batra *et al.*, 1993). Indeed, recent work has indicated that both the CH2 domain and the CH3 domain, contain sequences that control the serum persistence of IgG molecules (Kim *et al.*, 1994a; Pollock *et al.*, 1990, Kim *et al.*, 1994c; Medesan *et al.*, 1997). In particular, site-directed mutagenesis has been used to identify amino acid residues in the CH2-CH3 domain interface that are critical for the maintenance of serum IgG1 levels in mice (Kim *et al.*, 1994a; Medesan *et al.*, 1997), and this study therefore resulted in the precise localization of the catabolic site. These residues are highly conserved in both human and murine IgG isotypes (Kim *et al.*, 1994a; Table I), suggesting that the catabolic sites of human and murine IgGs are the same. The effects of two double mutants (HQ-310, His310 to Ala and Gln311 to Asn; HN-433, His433 to Ala and Asn434 to Gln), rather than single mutations at these positions, and a single mutation (Ile253 to Ala253) on catabolism and intestinal transfer have been characterized (Kim *et al.*, 1994a; Kim *et al.*, 1994b). In a more recent study (Medesan *et al.*, 1997) the effects of mutation of His310 to Ala310, His435 to Ala435, His436 to Ala436, His433 to Ala433, Asn434 to Ala434 or Gln434 have been analyzed.

Table I

Sequences of murine and human IgGs in the region of the catabolic site

	252-254	308-312	433-436**
mIgG1*	TIT	IMHQD	HNHH
mIgG2a	MIS	IQHQD	HNHH
mIgG2b	MIS	IQHQD	KNYY
mIgG3	MIS	IQHQD	HNHH
hIgG1*	MIS	VLHQD	HNHY
hIgG2	MIS	VVHQD	HNHY
hIgG3	*MIS*	*VLHQD*	*HNRF*
hIgG4	MIS	VLHQD	HNHY

* mIgG1 = murine IgG1, * hIgG + human IgG1

**His 433 and Asn 434 as a double mutant had an effect, but as single mutations, his 433 to ala 433 and asn 436 to ala 436, no effect was observed (Medesan *et al.*, 1997). Residues that were mutated and found to affect clearance rate (Kim *et al.*, 1994a) are underlined.

Mutation of His435 to Ala435 has a drastic effect on both catabolism and transcytosis, whereas mutation of His436 to Ala436 has a lesser effect (Medesan *et al.*, 1997). Mutation of only His310 to Ala310 has the same effect as mutating both His310 to Ala310 and Gln311 to Asn311, suggesting that Gln311 is not involved in the Fc:FcRn interaction. Individual mutation of His433 to Ala and Asn434 to Ala/Gln has no effect on binding to FcRn catabolism or transcytosis whereas in earlier studies (Kim *et al.*, 1994a; 1994c) it was noted that double mutation of His433,Asn434 did have a moderate effect. This variation is due to the perturbation of adjacent critical residues such as His435 by the double mutation (whereas single mutations are less perturbing) rather than direct involvement of 433 and 434 in the Fc:FcRn interaction.

Other residues in addition to Thr252, Thr254, Thr256, Met309 and Asn315 that might be useful targets for mutagenesis are Gln311, His433 and Asn434. Furthermore, data disclosed herein indicate that it is not valid to say that Gln311, His433 or Asn434 constitute the catabolic site, although double mutation of His433 and Asn434 does have an effect on catabolism.

Removal of the carbohydrate residues from the CH2 domain has a minor or no effect on the *in vivo* half life of IgGs, and the extent of this effect is dependent on the isotype (Nose and Wigzell, 1983; Tao and Morrison, 1989; Wawrzynczak *et al.*, 1989). The region of the Fc that is involved in the catabolism of IgG (Kim *et al.*, 1994a) appears to be distinct from the sites involved in binding FcγRI, RII and RIII receptors (the 'classical' FcRs), as these recognize sequences primarily located in the lower hinge region (Duncan *et al.*, 1988; Lund *et al.*, 1992; Sarmay *et al.*, 1992; Jefferis *et al.*, 1990; Canfield and Morrison, 1991; Wawrzynczak *et al.*, 1992). In addition, the catabolic site is distinct from the complement factor C1q binding site (Glu318, Lys320 and Lys322) (Wawrzynczak *et al.*, 1992; Duncan and Winter, 1988), thus mutation of the catabolic site should neither affect complement fixation nor binding to FcγRI, RII and RIII.

IgG2b and other murine isotypes

Murine IgG2b has been shown to have a more rapid clearance rate than IgG1, IgG2a and IgG3 (Pollock *et al.*, 1990). Analysis if sequence differences for the residues at the CH2-CH3 domain interface that have been shown to be important in building the catabolic site indicate that in IgG2b, His433, His435, His436 of IgG1, IgG2a and IgG3 are replaced by Lys433, Tyr435 and Tyr436 in IgG2b (Table I). These sequences differences may account for the differences in clearance rates and neonatal transfer (McNabb *et al.*, 1976; Guyer *et al.*, 1976) that have been observed. In this respect, Scharff and colleagues (Pollock *et al.*, 1990) have shown that sequence differences in the CH3 domain of IgG2a and IgG2b are responsible for the faster clearance rate of IgG2b relative to IgG2a, but have not identified the residues involved. In addition, murine IgG2b is not transferred across neonatal intestine as efficiently as murine IgG1 (Guyer *et al.*, 1976). The sequence differences in the CH3 region of the murine isotypes (Table I) provide an ideal system to analyze the role of position 433, 435 and 436 in controlling catabolism and transcytosis. The conversion of his 433 to ala 433, tyrosine (tyr) 435 to his 435 and tyr 436 to his 436 in an IgG2b molecule results in a mutated IgG2b that has the same *in vivo* half life as murine IgG1. Furthermore, the faster clearance rate of human IgG3 relative to IgG1, IgG2 and IgG4 further indicates that residue 435 (Table I) is involved in regulating serum IgG levels.

Possible mechanism of IgG catabolism

The maintenance of serum IgG concentrations at a fairly constant level is of importance for effective immunity. Moreover, abnormally high (hypergammaglobulinemia) or low (hypogammaglobulinemia) serum IgG levels result in clinical symptoms. To be effective, the homeostatic mechanism that both senses and regulates serum IgG levels must be able to deal with continuous and variable production of IgG molecules by the B cells of the organism. How such homeostasis is brought about is as yet unclear, and several mechanisms have been proposed to account for the control of IgG levels in the serum (Brambell *et al.*, 1964; Brambell, 1966; Ghetie *et al.*, 1981). Clearly, any model must invoke a feedback system that is both sensitive and responsive to changes in serum IgG levels.

Brambell and colleagues (Brambell *et al.*, 1964; Brambell, 1966) have proposed that a limited number of cellular receptors (designated FcRc in this proposal) bind to and protect the IgG molecules from degradation. The bound and internalized IgG molecule is protected from proteolysis and subsequently released back into the intravascular pool, whilst the IgG molecules that are internalized without bound receptors are degraded. Thus, the cells that are responsible for IgG breakdown are paradoxically also proposed to be involved in protection of IgGs against breakdown (Brambell *et al.*, 1964; Brambell, 1966). The receptors are saturable, and consistent with this model in hypergammaglobulinemic individuals, intravascular IgG is degraded much more rapidly than in hypogammaglobulinemics. This concentration dependence of catabolic rates is called the concentration-catabolism phenomena. The receptor model also fits with recent data which shows that mutation of specific residues at the CH2-CH3 interface of the IgG1 molecule results in rapid intravascular clearance (Kim *et al.*, 1994a), suggesting that the mutations have resulted in loss of recognition by the 'protective' receptors.

The site of immunoglobulin clearance

The site(s) at which IgGs are catabolized and the proteases involved have yet to be characterized. Both liver and gastrointestinal tract have been shown to play a role in the catabolism of IgG (Covell *et al.*, 1986; Hopf *et al.*, 1976; Dobre and Ghetie, 1979) but neither organ, however, has been demonstrated to be the major site of catabolism. Therefore the possibility of diffuse catabolism throughout the body must be considered (Waldmann and

Strober, 1969). Such diffuse catabolism could occur in the endothelial system throughout the body since the cells of this system are in close contact with the intravascular pool and IgG constantly traverses the endothelial cells to enter the extravascular space. Recent data support the notion of diffuse catabolism with possible involvement of endothelial cells.

Transfer of IgG across membranes (transcytosis)

Intestinal transfer in newborns

The mechanisms involved in transfer of passive immunity from the mother to young (fetus/newborn) may share similarities with that involved in the control of catabolism as proposed by Brambell (1966) and supported by recent data. In rodents, intestinal transfer of IgG can occur for up to two weeks after birth and is the major route by which suckling rodents acquire maternal IgG (reviewed in Morris, 1978; Jones and Waldmann, 1972). Maternal-fetal transfer of IgGs across the yolk sac is a more minor route of transfer in rodents, in contrast to humans where maternal-fetal transfer is the only route.

An Fc receptor (FcRn) has been implicated in transfer of IgG from the colostrum or milk into the bloodstream of newborn rats and mice (Brambell, 1966; Rodewald, 1976). Consistent with its' role in neonates, the receptor FcRn is not expressed in the duodenum of adult rodents. Binding studies (Wallace and Rees, 1980; Rodewald *et al.*, 1983) of isolated rat brush borders show that there are two classes of Fc receptors of differing affinities, and data indicate that the higher affinity FcR is involved in transcytosis (Hobbs *et al.*, 1987; Rodewald and Kraehenbuhl, 1984). FcRn has been isolated from duodenal epithelial brush borders of suckling rates (Rodewald and Kraehenbuhl, 1984 ; Simister and Rees, 1985) and the corresponding genes cloned (Simister and Mostov, 1989a; Simister and Mostov, 1989b). This Fc receptor comprises a heterodimer of two polypeptides of 51 kDa and 14 kDa. Interestingly, the 14 kDa component is β2-microglobulin and the 51 kDa component is homologuous to the heavy chain of Class I MHC proteins. The protein can be expressed in high yields in recombinant form and has recently been analyzed by x-ray crystallography (Burmeister *et al.*, 1994a; Burmeister *et al.*, 1994b). The gene encoding murine FcRn has been isolated and shown to be highly homologous to that of rats (Ahouse *et al.*, 1993). Interestingly, both rat and murine FcRn also share homology with a recently isolated Fc

receptor derived from human placenta that is most likely involved in maternal-fetal transfer (Story *et al.*, 1994). Thus, the available data indicate that IgG transcytosis in rats, mice and humans are carried out by similar receptors and as a consequence share a common mechanism.

The proposed mechanism of trans-intestinal transport is that FcRn on the lumenal side of intestinal epithelial cells binds IgG at pH 6-6.5 (the pH of the intestinal lumen) and the IgG-FcRn complexes are transported across the cell to the basolateral surface where exocytosis occurs into the bloodstream of the newborn rodent. Association of IgG with FcRn as it trafficks through the cell is postulated to protect the IgG molecule from lysosomal degradation. The pH of the plasma (7.4) results in release of the bound IgG into the circulation. Analyses of the binding of FcRn to IgG (or Fc) show a pH dependence that is consistent with this model, with strong binding at pH 6-6.5 and very weak (if any) binding at pH 7.4 (Rodewald, 1976; Wallace and Rees, 1980). Using recombinant Fc fragments, it has been shown that murine FcRn interacts with a region of the murine IgG1 molecule that overlaps with that involved in catabolism control and encompasses Ile253, His310, Gln311, His433 and Asn434 (Kim *et al.*, 1994b). More recent data have shown the involvement of His435 and His436, and also that His433 and Asn434 (if mutated individually and not as double mutations), do not play a role in interacting with FcRn (Medesan *et al.*, 1997). In addition, single mutation of His310 to Ala310 has the same effect as double mutation of His310 to Ala310 and Gln311 to Asn311, indicating that Gln311 does not interact with FcRn (Medesan *et al.*, 1997). Similar conclusions have been drawn for rat FcRn from the x-ray crystallographic data and *in vitro* binding studies of Bjorkman and colleagues (Burmeister *et al.*, 1994a; Burmeister *et al.*, 1994b; Raghavan *et al.*, 1994). Furthermore, for intestinal transfer, data demonstrate that two FcRn sites per Fc are necessary (Kim *et al.*, 1994b), consistent with the report that the stoichiometry of binding of rat FcRn to Fc is 2:1 (Huber *et al.*, 1993). The involvement of His310, His435 and His436 of the IgG1 molecule in interacting with FcRn explains, in part at least, the pH dependence of the FcRn-Fc interaction (Kim *et al.*, 1994b; Raghavan *et al.*, 1993).

Transfer across murine yolk sac (maternal-fetal transfer)

Murine FcRn is expressed at high levels in both neonatal intestine and yolk sac (Ahouse *et al.*, 1993), and an FcR that is structurally similar to FcRn has also been isolated from rat yolk sac (Roberts *et al.*, 1993). These data, together with *in vivo* studies disclosed herein strongly suggest that maternal-fetal and intestinal transport are carried out by FcRn, although the cellular location for IgG binding to FcRn appears to differ in the two processes (Roberts *et al.*, 1993). In rats, the yolk sac FcR is located in vesicles in the apical and basolateral cytoplasm, and not on the lumenal surface of the yolk sac endodermal cells (Roberts *et al.*, 1993). The difference in location is believed to be necessary because the pH of the lumen surrounding the yolk sac is slightly basic (Roberts *et al.*, 1993), and the affinity of binding of FcRn to IgG is low at this pH (Hobbs *et al.*, 1987; Rodewald and Kraehenbuhl, 1984); thus, it has been suggested that maternal IgG is taken up by the yolk sac cells in a non-specific endocytotic step and then binds to FcRn in a slightly acidic endosomal compartment. Delivery of IgG into the fetal circulation is then proposed to occur in a similar way to that of intestinal transcytosis (Roberts *et al.*, 1993). Similarly, with respect to the control of IgG catabolism, IgGs may be taken up by the 'catabolic' cells in a non-specific endocytotic step and subsequently bind to FcRn in an endosomal compartment.

Interrelationship between IgG transcytosis and control of catabolism

Data (Kim *et al.*, 1994a; Kim *et al.*, 1994b) suggest that, as originally proposed by Brambell and colleagues (1966; Brambell, 1966), the Fc receptors involved in catabolism control, maternal-fetal transfer and intestinal transfer bind to the same site of murine IgG1 and are closely related, if not the same. In support of this hypothesis, expression analysis indicates that, in addition to high level expression of FcRn in murine yolk sac and neonatal intestine, FcRn is ubiquitously expressed at lower levels in murine heart, lung, liver, spleen and endothelial cells lines but not in T nor B lymphocytes.

It is expected that maternal transfer of passive immunity to infants will be improved if the affinity of the Fc:FcRn interaction is increased and serum persistence is lengthened. For enhanced serum persistence and maternal-fetal transfer of a therapeutic IgG, it is preferable to endow that IgG with a higher affinity for binding to the Fc receptors that are involved in the

processes. As a result, the higher affinity IgGs should be able to out-compete the high concentrations of endogeneous IgGs (5 mg/ml in mice and 10 mg/ml in humans).

D: Engineered Antibody Domains with Extended *In Vivo* Half Lives

The mechanisms involved in regulating the *in vivo* catabolism of IgG molecules are currently not well understood, although the Fc region is believed to contain sequences that are important for serum persistence of IgG (Spiegelberg and Weigle, 1965). As described herein and by Pollock *et al.* (1990), the CH2 domain and the CH3 domain have been shown to influence biological half life of IgGs.

It has been observed that Staphylococcal protein A (SpA)-IgG complexes are cleared more rapidly from serum than uncomplexed IgG molecules (Dima *et al.*, 1983). Results from X-ray crystallography studies have indicated that residues in the Fc-hinge region are involved in SpA binding (Deisenhofer, 1981). These distinct lines of information prompted the present inventors to mutate residues at the CH2-CH3 domain interface of the (above-described) recombinant Fc-hinge fragment derived from the murine IgG1 molecule and to investigate the catabolism of the resultant mutants.

Using this approach, several amino acid residues of the CH2 domain, Ile-253 and His-310 (and double mutation of His310, Gln-311 to Ala310, Asn311), and of the CH3 domain (the double mutation of His-433-Asn-434, single muations of His433 and Asn434, and the single mutations of His 435 and His 436) were changed by *in vitro* mutagenesis. The mutant proteins were then purified from recombinant *E. coli* cells and the pharmacokinetic parameters measured in mice. The results from such studies demonstrate that amino acid residues from the CH2 domain, and those from the CH3 domain, are directly involved in the catabolism of mouse IgG1. Thus, the site of the IgG1 molecule that controls catabolism is located at the CH2-CH3 domain interface and is distinct from the lower hinge region that is involved in binding to Fc receptors (Duncan *et al.*, 1988; Lund *et al.*, 1991). The identification of specific amino acid residues that are involved in catabolism control supports the hypothesis that receptor bearing cells may be important in regulating serum IgG levels (Brambell *et al.*, 1964).

The inventors have termed the specific residues of the murine IgG1 molecule that they discovered to be involved in controlling the catabolism of this isotype the 'catabolic control site'. This region is distinct from the sites of interaction with classical Fc receptors (FcγRI, FcγRII, and FcγRIII) but overlaps with the SpA binding site. This is, therefore, consistent with earlier data that showed that SpA-immunoglobulin complexes were cleared more rapidly than uncomplexed immunoglobulins (Dima *et al.*, 1983). This data does not rule out the involvement of additional residues of the Fc fragment in catabolism control, but it does provide a clear means by which the biological half life of an antibody or antibody-based molecule or conjugate may be shortened. It also provides a means by which the longevity of a particular antibody may be increased if desired, by re-inserting residues such as ile253, his310, his435 and his436, should any such residues be found to be different in a particular antibody, *e.g.* IgG2b. Also random mutagenesis of residues flanking these key amino acids, followed by selection, may yield an Fc fragment with increased half life.

Although the mechanisms involved in the catabolism of IgG molecules have still to be completely elucidated, the data presented herein support the concept that SpA-like 'protective' receptors bind to the CH2-CH3 domain interface on IgGs and protect them from degradation. The engineered Fc-hinge fragments which form these aspects of the present invention are envisioned to be useful reagents in a variety of embodiments. For example, they may be employed in the isolation the putative receptor, which is most likely FcRn, and in further delineating the sites and mechanism of IgG catabolism.

The recombinant Fc-hinge fragments may also be useful for the preparation and delivery of immunotoxins where it is desirable to modulate the persistence of an immunotoxin in an animal. Immunotoxins are agents which have an antibody component linked to another agent, particularly a cytotoxic or otherwise anticellular agent, having the ability to kill or suppress the growth or cell division of cells. The preparation of immunotoxins is generally well known in the art (see, *e.g.*, U.S. Patent 4,340,535, incorporated herein by reference).

E: **FcRn**

Despite the central role that gamma-globulin (IgG) plays in immunity, little is known about the molecular mechanisms and dynamics by which remarkably constant IgG levels are maintained in the serum. Understanding the processes that maintain IgG homeostasis at the molecular level is of relevance to the treatment of IgG deficiencies and the effective delivery of therapeutic antibodies. Functional studies in neonatal mice indicate that the same amino acids of murine IgG1 (mIgG1) that regulate IgG catabolism, Fc residues Ile253, His310, His433, Asn434, His435 and His 436, where His433 and Asn434 are a double mutation, are also involved in binding to the MHC Class I homologue, FcRn, and this is consistent with the X-ray crystallographic structure of a rat FcRn: Fc complex (Burmeister *et al.*, 1994a, b). Rodent FcRn has been implicated in passive transfer of IgGs from mother to young primarily *via* neonatal transcytosis (Rodewald and Kraehenbuhl, 1984 ; Simister and Rees, 1985), and comprises a 45-50 kDa α-chain associated with β2-microglobulin (β2m; Simister and Mostov, 1989b). The effects of mutation of Ile253, His310, His433, Asn434, His435 and His436 on the B physiological half life of recombinant Fc-hinge fragments and on neonatal transcytosis correlate closely. This suggests that FcRn, or a closely related protein, might be the as yet unidentified Fc receptor that was originally suggested by Brambell and colleagues to be involved in regulating serum IgG levels (Brambell *et al.*, 1964). Such Fc receptors were proposed to maintain IgG homeostasis by binding and releasing IgGs back into the circulation and when IgG reaches saturating concentrations for the receptors, excess IgG is destined for degradation (Brambell *et al.*, 1964).

The catabolism of IgG is a diffuse process occurring not only in specific organs such as liver (Fukumoto and Brandon, 1979) and intestine (Covell *et al.*, 1986), but also in tissues containing reticulo-endothelial components such as spleen, skin and muscle (Mariani and Strober, 1990). Paradoxically, these cells may also bear the putative Fc receptors that recycle IgGs (Brambell *et al.*, 1964). The ubiquitous expression of rat FcRn (Simister and Mostov, 1989a) and a human FcRn homologue (Story *et al.*, 1994) outside the cells involved in maternofetal/neonatal transfer of IgGs would be consistent with a role in controlling IgG levels at sites throughout the body.

As described in Example 7, the expression of FcRn in mouse tissues and cell lines has been analyzed using reverse transcriptase (RT)-PCR™. FcRn α-chain mRNA is ubiquitously distributed in adult tissues/cell types, particularly those that are rich in endothelial cells. The pharmacokinetics of Fc-hinge fragments in genetically manipulated mice that lack FcRn expression (Zijlstra *et al.*, 1990; Koller *et al.*, 1990) due to disruption of the β2m gene (β2m-/- mice) have also been analyzed. The data support the involvement of FcRn in regulating IgG catabolism.

In the study described in Example 7, the analysis of the pharmacokinetics of IgG1/Fc fragments in β2m-/- mice provides evidence in support of the concept that the β2m dependent protein (Zijlstra *et al.*, 1990), FcRn, might be involved in maintaining serum IgG levels. The suggested implication of FcRn in this role is consistent with the ubiquitous expression of FcRn or its homologue in rats (Simister and Mostov, 1989a), man (Story *et al.*, 1994) and mice (FIG. 4A-FIG. 4C) and, in addition, the close overlap between the region of IgG involved in controlling neonatal transcytosis, IgG catabolism and binding to recombinant FcRn (Kim *et al.*, 1994b; Popov *et al.*, 1996; Medesan *et al.*, 1997).

RT-PCR™ analyses demonstrate that FcRn is expressed in liver, spleen and lung, but not in clonal B and T cell lines/hybridomas. Further analyses of expression in both mouse endothelial cell lines and hepatocytes indicated that FcRn is also expressed in these cell types. Quantitative PCR™ indicates that the level of expression in these cells is substantially lower than that in neonatal brush border, and this may account for the lack of detection of mouse FcRn α-chain mRNA in tissues other than neonatal brush border and yolk sac that was previously reported using Northern blotting (Ahouse *et al.*, 1993). Direct binding studies with the endothelial cell line SVEC indicate that WT Fc-hinge binds at significantly higher levels than the HQ-310/HN-433 mutant. Earlier observations demonstrating that the mutant Fc-hinge fragment binds at background levels to isolated neonatal brush border (Kim *et al.*, 1994b) and undetectably to recombinant FcRn (Popov *et al.*, 1996), suggest that the differential binding is mediated by FcRn. The possibility that the differential binding is due to interaction with FcgRI, II and/or III is made unlikely by reports which demonstrate that the interaction site of these receptors on Fc is distal to the CH2-CH3 domain interface (Duncan

et al., 1988; Canfield and Morrison, 1991; Lund *et al.*, 1991) and, furthermore, that aglycosylated Fc fragments are impaired in binding to these receptors (Tao and Morrison, 1989; Nose *et al.*, 1990). The binding data therefore suggest that FcRn is functional in SVEC cells, and is contemplated to be functional in the other cell types in which FcRn α-chain mRNA is expressed. Functional FcRn has also been isolated from SVEC cell lysates using murine IgG1 (mIgG1) coupled to Sepharose.

The functional significance of the expression of FcRn in both endothelial cells and hepatocytes suggests that either or both of these cell types might be involved in maintaining IgG homeostasis. In this respect, FcRn has been detected by immunoprecipitation from rat hepatocytes, and a role in mediating the trafficking of IgG into the biliary tract has recently been suggested to be of relevance for immunosurveillance at this site (Blumberg *et al.*, 1995). A distinct function for hepatocytic FcRn, however, might be that this protein sequesters bound IgGs from delivery into the bile and only unbound (excess) IgG is delivered for catabolism in the biliary tract. This is consistent with data indicating that IgG is delivered *via* liver cells into the bile for breakdown in sheep (Fukumoto and Brandon, 1979). Taken together with the earlier data of others (reviewed in Mariani and Strober, 1990; Zuckier *et al.*, 1989), however, the findings in this study support the involvement of both the liver and the more diffusely located endothelial cells.

The pharmacokinetic data demonstrate that mIgG1 or WT Fc-hinge have abnormally short serum half lives in β2m-/- mice. These serum half lives are not due to some generalized defect in the maintenance of serum Ig levels, as the serum half life of IgA is the same in both β2m+/+ and β2m-/- mice. Many studies have indicated that there is an inverse correlation between serum IgG concentrations and half lives of IgG and this is called the concentration-catabolism phenomenon (Waldmann and Strober, 1969; Zuckier *et al.*, 1989). The rapid elimination of mIgG1/WT Fc-hinge might therefore be due to abnormally high levels of endogenous serum IgGs in β2m-/- mice.

This is clearly not the case, however, as serum IgG levels are abnormally low in β2m-/- mice of both backgrounds, and these low serum IgG concentrations are consistent with the

observations of others (Spriggs *et al.*, 1992; Israel *et al.*, 1995). In contrast, the serum IgA and IgM concentrations, which are regulated by a mechanism distinct from that involved in IgG homeostasis (Strober *et al.*, 1968), are in the normal range.

To date, the role that IgG breakdown rates might have in mediating the low serum IgG concentrations in β2m-/- mice has not been investigated. It has previously been suggested that in normal mice, maternal IgG stimulates endogenous immunoglobulin synthesis, and lack of maternal transfer in β2m-/- mice accounts for the low IgG levels (Israel *et al.*, 1995). However, the data in this study show that although β2m-/- mice of both backgrounds have lower IgG1 synthesis rates, an additional cause of the low serum IgG levels is an increase in catabolic rates. The situation for mice of the C57BL/6 × 129/Ola background is made more complex by the observation that even for β2m+/+ animals of this background, IgG and IgG1 levels are abnormally low. This is due to a synthesis rate that, unexpectedly, is lower than that for β2m-/- mice of this mixed background. Thus, independently of the presence or absence of neonatal transfer of IgGs in mice of the mixed background, the IgG1 synthesis rate is abnormally low and the reasons for this are unknown. As a consequence of the low serum IgG concentrations in β2m+/+ mice of this background, and consistent with the concentration-catabolism phenomenon (Waldmann and Strober, 1969; Zuckier *et al.*, 1989), the half lives of mIgG1 and WT Fc-hinge are significantly longer in this strain than in β2m+/+ C57BL/6 mice.

The observations in β2m-/- mice are consistent with a model whereby β2m dependent Fc receptors, *i.e.* FcRn, which in normal mice regulate serum IgG levels, are either absent or dysfunctional in β2m-/- mice. However, alternative explanations cannot be excluded, particularly if loss of β2m is more pleiotropic than the currently available data indicate. This is made improbable by the presence of an apparently normal CD4+CD8- subset (Zijlstra *et al.*, 1990; Koller *et al.*, 1990) and the ability of B cells to mount T cell dependent antibody responses (Spriggs *et al.*, 1992; Mozes *et al.*, 1993) in β2m-/- mice. Other possibilities, such as either a deficiency in IgG-producing precursor cells or the absence of factors/cytokines produced by CD8+ cells resulting in the low serum IgG levels are excluded by the observations that β2m-/- mice have normal numbers of B220+/sIgM cells (Spriggs *et al.*, 1992) and, in Lyt2

knockout mice, lack of CD8+ cells does not result in reduced IgG levels (Fung-Leung *et al.*, 1991). In addition, the possibility that a β2m dependent protein similar to FcRn, rather than FcRn itself, is involved in IgG homeostasis is made unlikely by Southern blotting data indicating that in mice FcRn has no close homologue (Kandil *et al.*, 1995). However, it is conceivable that an unrelated, as yet unidentified, β2m dependent protein that binds to Fc or IgG at the same site as FcRn plays a role in maintaining serum IgG levels.

The ability of FcRn to bind and mediate the traffic of IgGs across neonatal intestinal and yolk sac cells suggests a mechanism by which FcRn in other tissues might protect IgGs against degradation by binding and recirculating it into the serum. Constant levels of FcRn expression would explain how IgG homeostasis is maintained despite variable IgG production by B cells, as once FcRn is saturated, excess IgG would be destined for degradation following endocytotic uptake (Brambell *et al.*, 1964). Concerning the site of FcRn-IgG complex formation, the pH dependence of this interaction (Rodewald and Kraehenbuhl, 1984 ; Simister and Rees, 1985; Gastinel *et al.*, 1992) suggests that for the maintenance of serum IgG levels, FcRn would bind to IgG following uptake by fluid phase endocytosis into intracellular, acidic compartments. This is in contrast to the FcRn-Fc interaction that occurs in the slightly acidic medium at the apical cell surface of jejunal epithelial cells during transcytosis across the neonatal intestine (Rodewald, 1973), but data in support of a similar mechanism for the maternofetal transfer of IgGs in both humans (Leach *et al.*, 1990) and rats (Roberts *et al.*, 1990) has been reported.

In summary, the findings suggest a new role for FcRn that is distinct from previously assigned functions (Rodewald and Kraehenbuhl, 1984 ; Simister and Rees, 1985), and this has relevance to understanding the molecular mechanisms that maintain IgG homeostasis. The sequence similarities between rodent FcRn and a recently identified human FcR (Story *et al.*, 1994) suggest that the present discovery will have implications for the therapy of IgG-related immunodeficiencies in humans and also for mediating maternal-fetal transfer of therapeutic IgGs across the human placenta.

F: Mutagenesis

In the present invention the mutagenesis of amino acid residues can either be random or site-specific. One may choose to make completely random mutations in the protein or alternatively to only randomly mutate certain residues as described in Example 4. One could also change a residue to any other amino acid residue; however, it is likely that certain residues would be preferred. For example, mutating hydrophilic residues that are essential to maintain the tertiary, or three-dimensional, structure of the protein to large hydrophobic residues would probably not be desirable since such mutations may destabilize the antibody and not extend the half life of the molecule. In addition, it would be preferred to mutate exposed residues as they are most likely to interact with FcRn.

Site-specific mutagenesis is a technique useful in the preparation of individual proteins or peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage or phagmid vectors such as pUC119. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, using the PCR™, which eliminates the step of generating single stranded DNA.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

Alternatively, PCR™ directed mutagenesis of double-stranded DNA can be used by designing oligonucleotide primers that overlap the site to be mutated. Such mutants may be readily prepared by, for example, directly synthesizing the Fc fragment by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference).

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

G: Vaccines

The present invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from engineered antibody Fc fragments. domains and/or peptides prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

The preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4.578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables. Either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week

intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

The following examples are intended to illustrate the practice of the present invention and are not intended to be limiting. As the invention is demonstrated with a variety of immunoglobulin-like domains, including murine antibody Fc-hinge, Fc, CH2-hinge and CH3 domains; and mutant domains with increased stability; it will be understood that other proteins or peptides will be adaptable to similar constructs as those described herein. Likewise, a variety of tags, linker sequences and leader sequences may be employed depending on the particular purification or isolation methods desired to obtain the polypeptide products.

EXAMPLE 1

The following example illustrates the production of an immunoglobulin Fc-hinge or Fc fragment and Fc-hinge or Fc derived subfragments in milligram quantities using *E. coli* as an expression host. These results indicate the suitability of the system for the commercial production of large quantities of recombinant protein.

Plasmids, Expression and Purification

PCR™ was used to isolate and tailor the genes encoding fragments derived from the murine IgG1 immunoglobulin molecule 9E10 (Honjo *et al.*, 1979; Evan *et al.*, 1985) for ligation into the expression plasmids (FIG. 1). To accomplish this, total RNA was extracted from 1×10^7 9E10 hybridoma cells, as described herein above. cDNA was primed using oligonucleotides CH3forBst or CH2forBst (see below; Honjo *et al.*, 1979) for the isolation of either the CH3 domain gene/Fc fragment genes or the CH2 domain gene respectively. The genes were then isolated using PCR™ and the primers shown below. As listed, the five distinct sequences represent SEQ ID NO:1 through SEQ ID NO:5, respectively.

a) CH3 domain,

CH3bakNco = 5' ATC ACC ATG GCC *GGC AGA CCG AAG GCT CCA CAG 3'*;

CH3forBst = 5' TAC AGG TGA CC*T TAC CAG GAG AGT GGG AGA GGC T 3'*

b) CH2-hinge,

HingebakNco = 5' ATC ACC ATG GCC *GTG CCC AGG GAT TGT GGT TG 3'*

CH2forBst = 5' ATC AGG TGA CC*T TGG TTT TGG AGA TGG TTT T 3'*

c) Fc fragment,

CH2bakNco = 5' ATC ACC ATG GCC *GAA GTA TCA TCT GTC TTC ATC 3'*

CH3forBst; as above

d) Fc-hinge fragment,

HingebakNco and CH3forBst; both as above.

A typical PCR™ comprised: 3 units Promega Taq polymerase, 10µl Promega buffer, 10 ml 0.2mM dNTP cDNA synthesis reaction in a final volume of 100 ml. Cycling conditions were: 94°C (0.5 min), 55°C (0.5 min), 72°C (1 min) for thirty cycles using a Techne temperature cycling block. The oligonucleotides each encode either an *NcoI* or *Bst*EII restriction site indicated by underlining, and italicized sequences) indicate the regions of the oligonucleotides that anneal to murine IgG1 constant region genes (Honjo *et al.*, 1979); which allows restriction digestion of the PCR™ products followed by gel purification and ligation as *Nco*I-*Bst*EII fragments into Vape1βHis (Ward, 1992). The ligated DNA was then transformed into *E. coli* BMH 71-18, as described above. The sequences of the inserts of all plasmid constructions were analyzed using the dideoxynucleotide method and either Sequenase (USB) for single stranded DNA templates and Femtomole kits (Promega) for double stranded DNA templates.

These antibody fragments can be expressed and secreted from recombinant *E. coli* cells, and the carboxy-terminal His_6 peptide tags allow purification using Ni^{2+}-NTA-agarose. *E. coli* BMH 71-18 transformants harboring the plasmids shown in FIG. 1 were grown up and induced for expression as described herein above. The recombinant proteins were isolated from the periplasm by osmotic shock followed by affinity purification using Ni^{2+}-NTA-agarose. The recombinant fragments were purified in yields of 2, 1-1.5, 1.5-2 and 0.5-1 milligrams per litre of culture for the CH3 domain, CH2-hinge fragment, Fc fragment and Fc-hinge fragment respectively. The purity of the recombinant proteins was assessed using SDS gel electrophoresis (Laemmli) and staining with Coomassie blue R-250.

CH2-hinge fragments were expressed as a mixture of dimers and monomers. dimers were separated from monomers using a Sepharose-G75 column (Pharmacia, Piscataway, NJ). Monomeric CH2-hinge fragments were prepared from dimers by reduction (using dithiothreitol) followed by treatment (blockade) of reduced sulfhydryl groups with iodoacetamide as described (Kim *et al.*, 1994c).

***In vitro* Analyses**

Results from HPLC analyses indicated that the CH3 domain, Fc fragment and Fc-hinge fragment are all expressed and purified as homodimeric proteins. For the Fc and CH3 domain, the dimers are non-covalently linked, as demonstrated by analyses on non-reducing PAGE (FIG. 2B). The dimerization of the Fc fragments and CH3 domains is presumably stabilized by non-covalent interactions between the CH3 fragments, which are closely apposed in the immunoglobulin structure (Marquart *et al.*, 1980). For the Fc-hinge dimer, the fragments are also covalently linked by -S-S- bridges between the hinge region cysteines.

In contrast, analysis of the CH2-hinge fragment using HPLC indicates that approximately 10% of the protein is expressed and purified as a dimer, and the remainder as monomers. Structural analyses of immunoglobulins indicate that the CH2 domains in the Fc regio: · f an antibody molecule form few interactions, and presumably the relative weakness of these interactions (compared with those between CH3 domain, for example) result in a low proportion of expressed dimers. The dimers are covalently linked by -S-S- bridges; expression

differences at positions 435 and 436 on maternofetal or neonatal transfer. In contrast to IgGs, IgM, IgE, and IgA have short serum half-lives and are not transferred across the placental/yolk sac barrier or neonatal gut (Zuckier *et al.*, 1989; Wild, 1973). Consistent with these observations, none of the residues shown in this study to be important for mediating the Fc-FcRn interaction are present in IgM, IgE, and IgA, although these three Ig classes of both humans and mice share significant homology with IgGs in other regions of the respective molecules (Kabat *et al.*, 1991).

I253 is a highly exposed, hydrophobic residue that is conserved in all IgG molecules belonging to mammals (Kabat *et al.*, 1991). In this study mutation of this isoleucine to alanine resulted in considerable decreases in the serum half-life and transcytosis across the maternofetal barrier or neonatal intestine. This clearly indicates that I253 fulfills a key physiological role beyond binding to SpA (Deisenhofer, 1981). The amino acid residues flanking I253 are involved in the binding of human Fc to SpA (Deisenhofer, 1981), and their participation in the binding of FcRn cannot be excluded. Thus, M252 is highly conserved in all IgG isotypes of mouse, rat, guinea pig, rabbit, and human with a few exceptions, such as mIgG1 and rat IgG1/IgG2a, for which threonine replaces methionine (Kabat *et al.*, 1991). Similarly, position 254 is occupied by serine for all isotypes and species except the above-mentioned mouse and rat isotypes that have threonine at this point. These changes in positions 252 and 254 may correlate with longer half-life and more efficient transcytosis of mIgG1 compared with the other isotypes.

For all mutants except H433A and H436A, binding to FcRn and SpA is impaired to a similar degree. H433A has reduced SpA binding relative to WT Fc hinge, but is unaffected when interacting with FcRn. Conversely, the H436A mutation has the opposite effect. Thus, although the SpA and FcRn interaction sites overlap, the overlap is not complete and the "footprints" of SpA and FcRn on mIgG1 are distinct. This is also consistent with the differences in pH dependence that are observed for the FcRn-mIgG1 and SpA-mIgG1 interactions (Wallace and Rees, 1980; Rodewald and Krachenbuhl, 1984; Ey *et al.*, 1978).

The pH dependence of the interaction between IgG and FcRn (binding at pH 6-6.5 and release at pH 7-7.5) (Wallace and Rees, 1980; Rodewald and Kraehenbuhl, 1984) falls in the range of the pK value of the imidazole side chains of histidine. Taken together with the data from this study, this information suggests that the marked pH dependence of the IgG-FcRn interaction

is determined by the surface accessible histidine residues at positions 310, 435, and 436 located at the interface of the CH2 and CH3 domains and this conclusion is in accord with data of Bjorkman and colleagues, indicating that for mIgG2a there are three titratable residues in the pH range of 6.4 to 6.9 (Raghavan *et al.*, 1995). Consistent with these studies (Raghavan *et al.*, 1995), analysis of the H310A mutant demonstrates that H310A plays a role in mediating the Fc-FcRn interaction both *in vitro* and *in vivo*. In contrast, however, analysis of H433A and H435A shows that for mIgG1, mutation of H435 to alanine results in a loss of affinity for FcRn, whereas H433 does not play a role in FcRn binding. Furthermore, mutation of H436 to alanine results in an Fc hinge fragment that has reduced affinity for FcRn. Thus, the histidines that play a role in mediating the high affinity of the mIgG1-FcRn interaction are H310, H435, and, to a lesser extent, H436. The reasons for the apparent differences in H433 and H435 between these data and those of others (Raghavan *et al.*, 1995) are not clear, but in the latter study different isotypes (mIgG2a, mIgG2b, and human IgG4) with consequent sequence differences in the residues both at and in proximity to the FcRn interaction site were used. Thus, it is conceivable that in the context of differences in the sequences of surrounding residues, the relative roles of H433 and H435 are distinct in different isotypes.

The close correlation between the effect of mutations of the Fc hinge fragments on pharmacokinetics, transcytosis across neonatal brush border/yolk sac, and affinity for FcRn (Table XX) supports the concept that FcRn is involved in all these processes (Ghetie *et al.*, 1996; Junghans and Anderson, 1996). This is also consistent with studies showing that in mice lacking FcRn due to loss of β_2m expression, IgGs have decreased intestinal transmission (Israel *et al.*, 1995; Zijlstra *et al.*, 1990) and abnormally short serum half-lives (Ghetie *et al.*, 1996; Junghans and Anderson, 1996). For both the control of catabolism and transcytosis, it has been hypothesized that only the IgG molecules bound to FcRn are protected from degradation and reenter the circulation (catabolism) or traverse the yolk sac/neonatal intestine (transcytosis) (Brambell *et al.*, 1964). FcRn was first identified as a functional protein in tissues of different species (placenta, yolk sac, and brush border of neontal intestine) involved in the transmission of antibody from mother to fetus or neonate (Wallace and Rees, 1980; Rodewald and Krachenbuhl, 1984; Roberts *et al.*, 1990; Simister *et al.*, 1996; Kristoffersen and Matre, 1996; Leach *et al.*, 1996). More recently, mouse FcRn α-chain mRNA has been isolated from organs not involved in

maternal transmission of IgGs, such as liver, lung, heart, and spleen (Ghetie *et al.*, 1996). Rat and human homologues of FcRn have also been found to be ubiquitously expressed at the mRNA level (Story *et al.*, 1994; Simister and Mostov, 1989b; Blumberg *et al.*, 1995). This strongly suggests that FcRn might be synthesized by the endothelial cells within these organs. Consistent with this, FcRn α-chain mRNA (Ghetie *et al.*, 1996) and the corresponding protein have been isolated from cultivated mouse endothelial cells (SVEC), suggesting that endothelial cells might be the site of IgG catabolism. The isolation of a human homologue of FcRn from human placenta (Simister *et al.*, 1996; Kristoffersen and Matre, 1996; Leach *et al.*, 1996) that is ubiquitously expressed in adult tissues (Story *et al.*, 1994) together with the high degree of conservation of I253, H310, and H435 in human IgGs (Kabat *et al.*, 1991) indicate that the same mechanisms of maternofetal transfer and homeostasis of serum IgGs are operative in humans. Understanding these processes in molecular detail has implications both for the modulation of the pharmacokinetics of therapeutic IgGs and for the enhancement of maternofetal transfer of IgGs that might be of value in passive immunization of fetuses.

TABLE XX
Pearson's Correlation Coefficient Test

	Catabolism	Intestinal Transfer	Affinity for FcRn	Protein A Binding
Maternofetal	r=0.8703	r=0.8928	r=0.8358[a]	r=0.8838
Transmission	p=0.0049	p=0.0028	p=0.0097	p=0.0036
Catabolism		r=0.9450	r=0.9776	r=0.7107
		p=0.0004	p=0.00003	p=0.0482
Intestinal Transfer			r=0.9531	p=0.8361
			p=0.00025	p=0.0097
Affinity for FcRn				r=0.7709
				p=0.0251

[a]Correlation coefficient excluding values obtained for H436A; r=0.9917; p=0.00001.

The pH dependence of the FcRn-IgG interaction (Wallace and Rees, 1980; Rodewald and Krachenbuhl, 1984) suggests that the subcellular site (cell surface or intracellular compartment) at which binding occurs differs for neonatal transcytosis and maternofetal transfer/control of catabolism, as discussed previously (Ghetie *et al.*, 1996). Other unknown factors, such as the rate of recycling in these different cellular compartments, may also play a role in determining the effective concentration of FcRn. These differences between the processes and the cell types involved, despite the involvement of a common receptor, may explain the behavior of the H436A mutant, for which the half-life, intestinal transfer, and affinity for FcRn do not correlate with the maternofetal transmission as closely as for the other mutants. A further explanation for the anomalous effects of the H436A mutation might be as follows: mutation of H436 to alanine does not have as marked an effect on catabolism, inhibition of intestinal transfer, and binding to FcRn as those observed for I253A, H310A, and H435A, and in contrast to the other three assays, the maternofetal transfer assay is conducted in the absence of competition by endogenous IgGs using SCID mice. Thus, in this situation the effect of this mutation on maternofetal transfer only manifests itself if an analysis of the time course of transmission is conducted or if transfer is

analyzed in the presence of endogenous competing IgGs in, for example, BALB/c mice. In contrast, for mutants such as I253A, H310A, and H435A that have lower affinity than H436A for binding to FcRn in competition assays, the low activities in all three *in vivo* assays (catabolism, maternofetal transfer, and inhibition of neonatal transcytosis) correlate closely.

In summary, this study has resulted in the unequivocal identification of a role for three highly conserved histidines of mIgG1 (H310, H435, and, to a lesser degree, H436) in the control of catabolism and maternofetal/neonatal transcytosis. Thus, taken together with earlier data implicating I253 in these processes, these residues are critical for the FcRn-mIgG1 interaction. This study further extends the evidence in support of the involvement of FcRn in both transcytosis and catabolism, and has relevance to understanding the molecular mechanisms that regulate these essential functions of IgGs.

EXAMPLE 10

The power of bacteriophage display for the affinity improvement of antibodies for binding to cognate antigen has already been demonstrated. In this study, the system will be used to express mutated Fc fragments and to select for higher affinity variants for binding to FcRn. For this work, milligrams quantities of soluble FcRn (sFcRn) are used and the WT Fc fragment is expressed in functionally active form on the surface of phage.

Strategy for mutagenesis

Previous work has indicated that Ile 253, His310, His433,Asn434, His435 and His436 play a role, either directly or indirectly, in binding to FcRn (Kim *et al.*, 1994a; 1994b; Medesan *et al.*, 1997 and Example 9). Residues flanking these key residues, and have side chains that, from the X-ray structure of human IgG1 (Deisenhorfer, 1981), are most likely exposed in the vicinity of the CH2-CH3 domain interface were selected for random mutagenesis. Furthermore, these residues should not be conserved within and across species (*e.g.* in mice compared with humans), suggesting that they are not (directly) involved in catabolism control and transcytosis (or some other important functions of IgGs). Examples of such residues for the murine IgG1 isotype are, in EU numbering (Edelman *et al.*, 1969), Thr252, Thr254, Thr256 (see Example 4 for analysis), Met309, Gln311, Asn315 in the CH2 domain and His433 and Asn434 in the CH3 domain.

Prior to random mutagenesis, the effect of mutating each of these residues to alanine on binding of the Fc fragment to sFcRn is analyzed. If any of these residue changes lower the affinity by more than 10-fold, they are not mutated randomly. However, most of these residues listed above are not highly conserved (Kabat *et al.*, 1991) and it is therefore believed that they are likely to play a key role in the Fc:FcRn interaction and by extension, in control of catabolism and IgG transcytosis. As His433 and Asn434 are highly exposed on a loop protruding from the CH3 domain (Deisenhofer, 1981), there are few flanking residues that would be preferred candidates for mutagenesis. A greater number of mutations are made in the CH2 domain than in the CH3 domain.

These residues are randomly mutated in groups of 2-6 (for example, Gln309/Arg315 random mutants are combined with Thr252, Thr254 and Thr256 random mutants) using oligonucleotides that match the flanking codons precisely but insert random bases in the codon positions corresponding to the residues mutated. For insertion of mutated codons that are distal to each other, within the same Fc gene, two rounds of PCR™ are used with two or more different oligonucleotides. The random bases in the oligonucleotides are designed so that the wobble position of the codon end in A and to avoid biases towards particular amino acids, C may also be removed from the wobble position (as described by Hoogenboom and Winter, 1992). Mutated genes are either assembled by splicing by overlap extension (Horton *et al.*, 1989) or by using unique restriction sites (if located in the proximity of the mutation site). Following mutagenesis, approximately 20 clones made using each mutagenic oligonucleotide are sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977). Expression levels of approximately 20 clones are analyzed using the anti-myc antibody 9E10 and immunoblotting as described previously (Ward *et al.*, 1989).

Selection of Mutants with Higher Affinity for Binding to FcRn

Two strategies for selection of higher variants from libraries of mutated Fc genes are used. In the first, mutated genes are assembled in pHEN1 (Hoogenboom *et al.*, 1991) and used to transfect *E. coli* TG1. As indicated above, the leakiness of suppression results in both soluble Fc and phage bound Fc fragments being exported from the recombinant cells, and these should assemble as homodimers on the surface of phage. Phage bearing Fc fragments ('Fc-phage') are

propagated, concentrated by polyethylene glycol precipitation and panned on sFcRn coated Dynabeads/Ni^{2+}-NTA-agarose as described previously (Marks *et al.*, 1991; Ward, 1977; Popov *et al.*, 1995). sFcRn are purified from baculovirus infected insect cells as described previously (Popov *et al.*, 1996). Rounds of panning followed by phage propagation should result in enrichment for higher affinity binders. In addition, to select for higher affinity variants, procedures analogous to that described by Winter and colleagues (Hawkins *et al.*, 1992) are used; first, phage are mixed with small amounts of soluble biotinylated sFcRn (<1 μg) such that the antigen is in excess over the phage but at a concentration lower than that of the dissociation constant that is required (7.8 nM; Raghavan *et al.*, 1994). sFcRn bound Fc-phage particles are then used, and Ni^{2+}-NTA-agarose added to separate sFcRn bound phage. Second, to select for Fc fragments with lower off rates, Fc-phage particles are preloaded with biotinylated sFcRn and then diluted in to excess unlabeled antigen for variable times prior to addition of streptavidin coated beads as described previously (Hawkins *et al.*, 1992). Alternaitvely, the selection method used in Example 4 is utilized.

All binding steps are carried out at pH 6 and bound phage eluted at pH 7.4 prior to infection of exponentially growing *E. coli* TG1 cells. Following several rounds of panning, the host strain are switched to the non-suppressor strain *E. coli* HB2151 (Marks *et al.*, 1991). The Fc variants are analyzed as soluble secreted Fc fragments in ELISAs with sFcRn coated plates, using biotinylated Fab fragments derived from the anti-myc tag antibody, 9E10, for detection (note, the complete 9E10 antibody is not used as this is a murine IgG1 antibody and competes with Fc fragments for binding to FcRn). For this, Fc fragments are purified from recombinant *E. coli* cells using the c-myc tag as an affinity purification tag (Marks *et al.*, 1991; Popov *et al.*, 1995); alternatively, the genes are recloned as *Nco*I-*Not*I fragments into a vector derivative of VβpelBHis (Ward, 1992) with an in-frame polyhistidine tag for purification using Ni^{2+}-NTA-agarose. For detection of His_6 tagged proteins, the Fc fragments are biotinylated prior to use (Amersham biotinylation kits). All binding steps in ELISAs are carried out at pH 6.

As the murine Fc fragment is expected to have a high affinity for binding to FcRn (by analogy with the rat FcRn:Fc interaction), it may be difficult to select variants with increased affinity for the FcRn:Fc interaction, particularly if there are two functional binding sites per Fc (increasing the avidity). Thus, an approach based on earlier Fc-hybrid work (Kim *et al.*, 1994c) is

taken. The Fc-hybrid comprises a heterodimer of one WT Fc polypeptide associated with one mutant (HQ-310/HN-433) and has a reduced binding affinity due to loss of avidity, as the HQ-310/HN-433 mutant binds FcRn poorly (Kim *et al.*, 1994b; Popov *et al.*, 1996b). Thus, co-expression of randomly mutated WT Fc fragments with HQ-310/HN-433 may facilitate the selection of higher affinity variants which bind strongly to FcRn as monomers. Such mutants can subsequently be expressed as homodimers and their affinities as bivalent fragments determined.

Randomly mutated Fc fragments (derived from WT Fc) are ligated into a modified version of pHEN1 that has the stop codon removed by site-directed mutagenesis (Zoller and Smith, 1982). Thus, following transformation into *E. coli all* random mutants are expressed as cpIII linked fusions. *E. coli* is also co-transformed with a plasmid made previously by ligating the HQ-310/HN-433 Fc gene into pBGS19 (a derivative of pUC119 that confers kanamycin resistance; Spratt *et al.*, 1986). Double transfectants are selected on ampicillin plus kanamycin plates (as described by Kim *et al.*, 1994c; Riechmann *et al.*, 1988) and phage propagated, concentrated by polyethylene glycol precipitation and panned on sFcRn coated Dynal beads/Ni^{2+}-NTA-agarose using the approaches for the selection for higher affinity variants described above.

To express higher affinity variants as soluble secreted proteins, the genes are recloned into pHEN1 and expressed using *E. coli* HB2151 as host. Alternatively, the genes are cloned into the VβpelBHis (Ward, 1992) derivative to allow purification using Ni^{2+}-NTA-agarose as above. ELISAs are also carried out as above.

Affinity Measurements of Higher Affinity Variants

Fc mutants with higher affinity for FcRn are purified from recombinant *E. coli* cells using the c-myc epitope or polyhistidine peptide as an affinity purification tag as described previously (Ward, 1992; Marks *et al.*, 1991; Popov *et al.*, 1995). The affinities of these Fc fragments for binding to sFcRn are determined using surface plasmon resonance (SPR) and the BIAcore (Karlsson *et al.*, 1991). This approach has been used to analyze antibody-antigen interactions (Ward, 1994; Borrebaeck *et al.*, 1992), and Bjorkman and colleagues have characterized the interaction of IgG with rat FcRn using SPR (Raghavan *et al.*, 1994; Popov *et al.*, 1996b).

Studies are carried out to determine both the equilibrium binding constant and the kinetic dissociation constant ($Kd = k_{off}/k_{on}$, where k_{off} and k_{on} are off and on rates, respectively). Purified sFcRn are directly coupled to a CM5 chip (Pharmacia) using the standard amine coupling procedure. For chemical coupling, sFcRn are coupled in 10 mM acetate buffer (pH 5.5) at 20 μg/ml. Initially, both types of sensor chip are used and the relative amount of correctly folded sFcRn bound are determined using either murine IgG1 or WT Fc. Fc fragments are transferred to BIAcore running buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM EDTA, 150 mM NaCl and 0.05% P20, pH 7.4) using either a desalting column or dialysis and initially used in a concentration range of 0.05-0.5 mg/ml. These fragments are also purified by size exclusion (HPLC) immediately prior to use to remove aggregates, as this is essential to avoid artifacts. Flow rates of 5-30 μl/min are used.

As an alternative assay format, Fc fragments are coupled to CM5 chips as above, and sFcRn in BIAcore running buffer is initially used in the concentration range of 0.05-0.5 mg/ml. Initial studies are carried out to analyze the interaction between sFcRn and complete IgG1/WT Fc and are then extended to analysis of the mutant Fc fragments that putatively have higher affinities.

Pharmacokinetics of maternal-fetal and intestinal transfer of Fc mutants

The methodology as described above in the preceding Examples is used.

Incorporation of higher affinity mutation into IgG2a

Of the murine isotypes, IgG2a is the most efficient at ADCC and also carries out complement mediated lysis effectively (Herlyn and Koprowski, 1982; Bindon *et al.*, 1988). The catabolic site residues Ile253, His310, His435 and His436 are also present in IgG2a (Table I) and consistent with this, the half lives of IgG1 and IgG2a are similar (Pollock *et al.*, 1990). Thus, to improve the transcytosis and serum persistence of this isotype, the same mutations that are found to result in improved transcytosis and serum persistence of IgG1 are incorporated into a recombinant Fc fragment derived from the Y-3P hybridoma (IgG2a; Janeway *et al.*, 1984). Recombinant Fc fragments are purified and analyzed using the same methods as those described in previous Examples.

Analyses of the effect of the mutations on complete glycosylated IgGs

The IgG1 isotype

A construct for the expression of the murine IgG1 antibody (anti-arsonate) is tailored using the PCR™ with suitable restriction sites for ligation into pRL1 (Riechmann *et al.*, 1988) and used to transfect myeloma NS0 cells. In addition, mutations of Fc fragments that result in higher affinity binding to FcRn, increased transcytosis and, possibly, improved serum persistence are incorporated into the Fc gene of this construct using site-directed mutagenesis (Zoller and Smith, 1982). All mutants are sequenced (Sanger *et al.*, 1977) prior to expression analysis to ensure that there are no second site mutations.

Stable transfectants are generated by electroporation of NSO cells followed by selection using mycophenolic acid as described previously (Riechmann *et al.*, 1988) or more preferably by using the baculovirus system to transfect insect cells as described in Example 8. This latter system is preferred as it is faster. For NSO cell trasnfections, clones are isolated by limiting dilution and the expression levels of individual clones is determined by pulsing the cells with ^{35}S-methionine followed by SDS-PAGE of culture supernatants. PAGE gels are dried down and autoradiographed. IgG1 mutants are purified using arsonate (Ars)-Sepharose or protein A-Sepharose (however, it is conceivable that the mutations that result in higher affinity binding to FcRn have a detrimental effect on protein A binding, and therefore the use of Ars-Sepharose may be preferable). The WT and mutant IgG1 antibodies are radiolabeled and used in pharmacokinetic, maternal-fetal transfer and intestinal transfer studies as described (Kim *et al.*, 1994a; Kim *et al.*, 1994c).

Other isotypes

The genes encoding murine isotypes (or mutant derivatives) other than IgG1 are used to replace the IgG1 constant regions in the expression construct described above. These constructs are used to transfect NS0 cells and Ars specific antibody purified using Ars-Sepharose or SpA-Sepharose. Alternatively, complete glycosylated IgGs are expressed in the baculovirus system as described in Example 8 for murine IgG2b. These antibodies are used in pharmacokinetic, maternal-fetal transfer and intestinal transfer studies as described above.

Mutations at the CH2-CH3 domain interface and effects on complement fixation and ADCC

If the mutant antibodies are to be used in therapy with their own functions (complement fixation and ADCC), it is important to ensure that if they bind to FcRn with higher affinity and, as a consequence, are transcytosed more efficiently, and have longer serum persistence, they also retain their original properties with respect to ADCC and complement fixation. Murine IgG2a and 2b are most effective at complement fixation and ADCC (Herlyn and Koprowski, 1982; Bindon *et al.*, 1988) and therefore it is particularly important to check that these have not lost activity. In contrast, murine IgG1 is relatively inactive in these two effector functions (Herlyn and Koprowski, 1982; Bindon *et al.*, 1988). The following assays are therefore carried out.

Complement mediated lysis

Methodology similar to that described by Duncan and Winter (1988) is used. Sheep red blood cells (RBCs) are washed in PBS and resuspended at 10^9 cells/ml. Arsonate is coupled to the cells by adding -p-azobenzenearsonate-L-tyrosine-t-Boc-N-succinimide using previously described methodology (Harlow and Lane, 1988). Cells are washed three times in complement fixation diluent (CFD) and resuspended at 10^8 cells/ml. Cells are labeled by incubation with $Na^{51}CrO_4$ (0.2 mls per ml of cells, specific activity, 1-10 mCi/ml) for 4 hours at 37°C. Serial dilutions of antibody (wild type and mutants) are made in CFD in flat bottom wells of 96 well plates. Fifty microliters of a 1:10 dilution of guinea pig serum and 100 µl derivatized labeled cells are added to each well. Results are expressed using the following formula:

$$\text{Specific } ^{51}\text{Cr release} = \frac{(\text{observed release} - \text{spontaneous release})}{\text{total incorporated activity}} \times 100$$

ADCC

Essentially the methodology of Sarmay and colleagues (1992) is used. Murine or chicken RBCs are derivatized with arsonate and pulsed with $Na^{51}Cr0_4$ as described above. Serial dilutions of WT and mutant antibodies are added to wells of 96 well plates (containing 10^5 cells/well) and effector cells added to the target cells in ratios ranging from 0.5-5 (effector:target). As effector

cells, mouse peritoneal macrophages or P388D1 cells activated with IFN-γ (Nathan *et al.*, 1983) are used. Cells are incubated for 12 hours at 37°C and specific ^{51}Cr release determined as above

EXAMPLE 11

This example describes Fab fragments which have much longer serum persistence than $F(ab')_2$ fragments that do not bind to FcRn. These fragments represent non-Fc ligands that bind to FcRn, with a range of affinities, and have longer serum persistence.

Isolation of $F(ab')_2$ fragments that have long serum persistence

New Zealand white rabbits were immunized subcutaneously with soluable, recombinant FcRn (Popov *et al.*, 1996b) using 100 μg FcRn emulsified in incomplete Freund's adjuvant for subsequent injections at two weekly intervals until a suitable anti-FcRn titer had been reached . Polyclonal sera was then isolated and IgGs purified using protein A-Sepharose (Pharmacia, Piscataway, NJ). The IgGs were digested with pepsin to generate $F(ab')_2$ and Fc fragments, and purified $F(ab')_2$ fragments separated from Fc and any undigested IgG using protein A-Sepharose. Anti-FcRn $F(ab')_2$ fragments were purified using FcRn-Sepharose and then absorbed using MHC class I expressing RMA cells to remove cross-reactive anti-MHC class I $F(ab')_2$ fragments. $F(ab')_2$ fragments that do not bind to FcRn ("control" $F(ab')_2$) were obtained from preimmune sera of rabbits using the same methods for purification of IgGs followed by digestion with pepsin. Both $F(ab')_2$ preparaions were radioloabeled with Iodogen, as described previously, and used in clearance studies in SWISS mice (3-4 mice per group). The β phase half lives were 24.6 ± 3.6 hours for the control $F(ab')_2$ and 78.2 ± 4.8 for the anti-FcRn $F(ab')_2$. Clearly the anti-FcRn $F(ab')_2$ fragments (or, most likely, a subset of the $F(ab')_2$ within the polyclonal population) had long serum persistence that approaches the β phase half life of murine IgG1 or Fc-hinge in the same strains (about 120 hours).

* * *

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More

specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All claimed matter can be made without undue experimentation.

REFERENCES

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques, and/or compositions employed herein.

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U.S. Patent 3,949,064.

U.S. Patent 4,174,384.

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(ii) TITLE OF INVENTION: IMMUNOGLOBIN-LIKE DOMAINS WITH INCREASED HALF LIVES

(iii) NUMBER OF SEQUENCES: 25

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATCACCATGG CCGGCAGACC GAAGGCTCCA CAG 33

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TACAGGTGAC CTTACCAGGA GAGTGGGAGA GGCT 34

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATCACCATGG CCGTGCCCAG GGATTGTGGT TG 32

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATCAGGTGAC CTTGGTTTTG GAGATGGTTT T 31

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATCACCATGG CCGAAGTATC ATCTGTCTTC ATC 33

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGGCTCCT CCGTGCT 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATCATCTAGA TTTTTTTGTT GGGGCCAAAT TTATG 35

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATCACCATGG CCGGTAGGAT GCGCAGCGGT CTGCCAGCC 39

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCAGTCGAC CTTGGAAGTG GGTGGAAAGG CATT 34

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:21..34
(D) OTHER INFORMATION:/note= "N = A, G, C or T"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:20
(D) OTHER INFORMATION:/note= "S = G or C"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAACACACGT GACCTTAGCS NNCAGSNNAA TSNNGAGC 38

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCACGTGTG TTG 13

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCTCCTCCCG GGGTTGCGT 19

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:22..23
(D) OTHER INFORMATION:/note= "N = A, G, C, or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CAGGAAGCTG ACCCCTGTGG GNN 23

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:22..23
(D) OTHER INFORMATION:/note= "N = A, G, C, or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTCCGTCTCA GGCCACTCCC CNN 23

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCAGGAAGTG GCTGGAAAGG CATT 24

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGGGGATGC CACTGCCCTG G 21

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGTGGTTGGC CAGGCCCCT 19

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CAGTATGGGC GTTGTGCA 18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CTCAGTAGCG TGGTTGTG 18

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCCATCATGG CCCAGGACTG G 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CCAGTCCTGG GCCATGATGG G 21

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGCCTGCACG CGCACCATAC T 21

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AGTATGGTGC GCGTGCAGGC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AGTATGGTGT TGGTGCAG 18

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CTGCACCAAC ACCATACT 18

CLAIMS

1. A composition comprising a mutant IgG molecule having an increased serum half-life relative to IgG, and wherein said mutant IgG molecule has at least one amino acid substitution in the Fc-hinge region.

2. The composition of claim 1, wherein said IgG is a human IgG.

3. A composition comprising a mutant IgG Fc-hinge fragment having an increased serum half-life relative to the serum half-life of IgG, and wherein said fragment has an increased binding affinity for FcRn.

4. A composition comprising a mutant IgG Fc-hinge fragment having an increased serum half-life relative to the serum half-life of IgG, and wherein said fragment has the same or slightly lower affinity than IgG for binding to FcRn.

5. The composition of claim 1 or claim 3, wherein said molecule or fragment has an amino acid substitution at one or more of the amino acids selected from number 252, 254, 256, 309, 311 or 315 in the CH2 domain or 433 or 434 in the CH3 domain.

6. The composition of claim 5, wherein said molecule or fragment has three amino acid substitutions at amino acid number 252, 254, 256, 309, 311 or 315 in the CH2 domain or 433 or 434 in the CH3 domain.

7. The composition of claim 6, wherein said molecule or fragment has the following amino acid substitutions: leucine for threonine at position 252, serine for threonine at position 254 and phenylalanine for threonine at position 256.

8. The composition of claim 1 or claim 3, wherein said molecule or fragment has a dissociation constant for binding to FcRn at pH 6, of less than about 7 nM as measured by surface plasmon resonance analysis.

9. The composition of claim 1 or claim 3 further defined as a pharmaceutically acceptable composition.

10. The composition of claim 5, wherein said amino acid substitutions are generated by random mutagenesis.

11. A method of increasing the serum half-life of an agent comprising conjugating said agent to a mutant IgG or IgG Fc hinge fragment having an increased serum half life of claim 1 or 3.

12. The method of claim 11, wherein said agent is a therapeutic drug.

13. The method of claim 11, wherein said agent is an antigen binding polypeptide.

14. The method of claim 11, wherein said agent is an antigen or a receptor binding ligand.

15. The method of claim 14, wherein said receptor binding ligand is a T-cell receptor binding ligand.

16. A method of making an antibody with an increased serum half life comprising:

identifying a first amino acid in an IgG hinge region that is suspected of being directly involved in FcRn binding;

identifying one or more second amino acids wherein each of said second amino acids is in the spatial region of said first amino acid, and wherein the side chain of said second amino acid is exposed to solvent in the native antibody;

making an antibody with a random amino acid substitution of one or more of said second amino acids to make a mutant antibody; and

identifying a mutant antibody having an increased serum half life.

17. The method of claim 16, further comprising the step of isolating said antibody.

18. The method of claim 16, wherein said first amino acid is amino acid number 253, 310, 435 or 436 of the Fc fragment.

19. The method of claim 16, wherein said second amino acid is amino acid number 252, 254, 256, 309, 311 or 315 in the CH2 domain or 433 or 434 in the CH3 domain.

20. The method of claim 16, wherein two or more of said second amino acids are mutated in a single mutant antibody.

21. An antibody having an increased serum half life, wherein said antibody is made by the method of claim 16.

22. A composition comprising an Fc fragment comprising the fragment from about amino acid 250 to about amino acid 440 of an IgG antibody, further defined as:

having a higher binding affinity for FcRn than said IgG antibody;

having one or more amino acid substitutions in a region near one or more FcRn binding amino acid residues; and

having a higher binding affinity for FcRn at pH 6 than at pH 7.4.

23. A method of decreasing endogeneous serum IgG in a subject comprising administering to said subject an effective amount of the composition of claim 22.

24. A method of screening an agent for an increased serum half-life relative to the serum half-life of IgG, comprising the steps of:

obtaining a candidate agent;

measuring the binding affinity of said agent to FcRn at pH 7.4 and at about pH 6;

selecting a candidate agent with a higher binding affinity for FcRn at about pH 6 than at pH 7.4; and

comparing the binding affinity of said selected agent to FcRn to the binding affinity of IgG to FcRn under identical conditions;

wherein an increased binding affinity for FcRn relative to the binding affinity of IgG is indicative of an agent with an increased serum half-life.

25. The method of claim 24, wherein said candidate agent is a peptide or polypeptide.

26. The method of claim 25, wherein said peptide or polypeptide is an antibody or a fragment of an antibody.

27. The method of claim 25, wherein said peptide is selected from a random peptide library.

28. The method of claim 25, wherein said polypeptide is a protein.

29. The method of claim 28, wherein said protein is a randomly mutated protein.

30. The method of claim 25, wherein said peptide is a synthetic peptide.

31. The method of claim 25, wherein said peptide is a chemical compound isolated from a random library of synthetic chemical compounds.

32. A method of increasing maternofetal transfer of either antibodies or drugs or proteins or other therapeutic agents comprising administering to said subject an effective amount of the composition of claim 22.

33. A method of increasing the serum half-life of a therapeutic agent comprising:

conjugating said therapeutic agent to an agent having an increased serum half-life relative to the serum half-life of IgG identified by the method of claim 23.

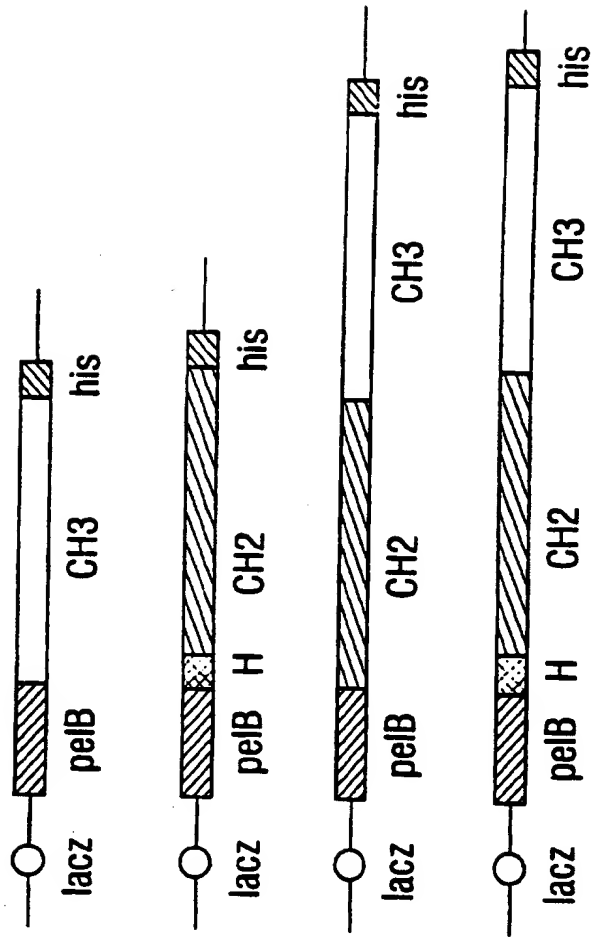
lacz
pelB
CH3
his
lacz
pelB
H
CH2
his
lacz
pelB
CH2
CH3
his
lacz
pelB
H
CH2
CH3
his

FIG. 1

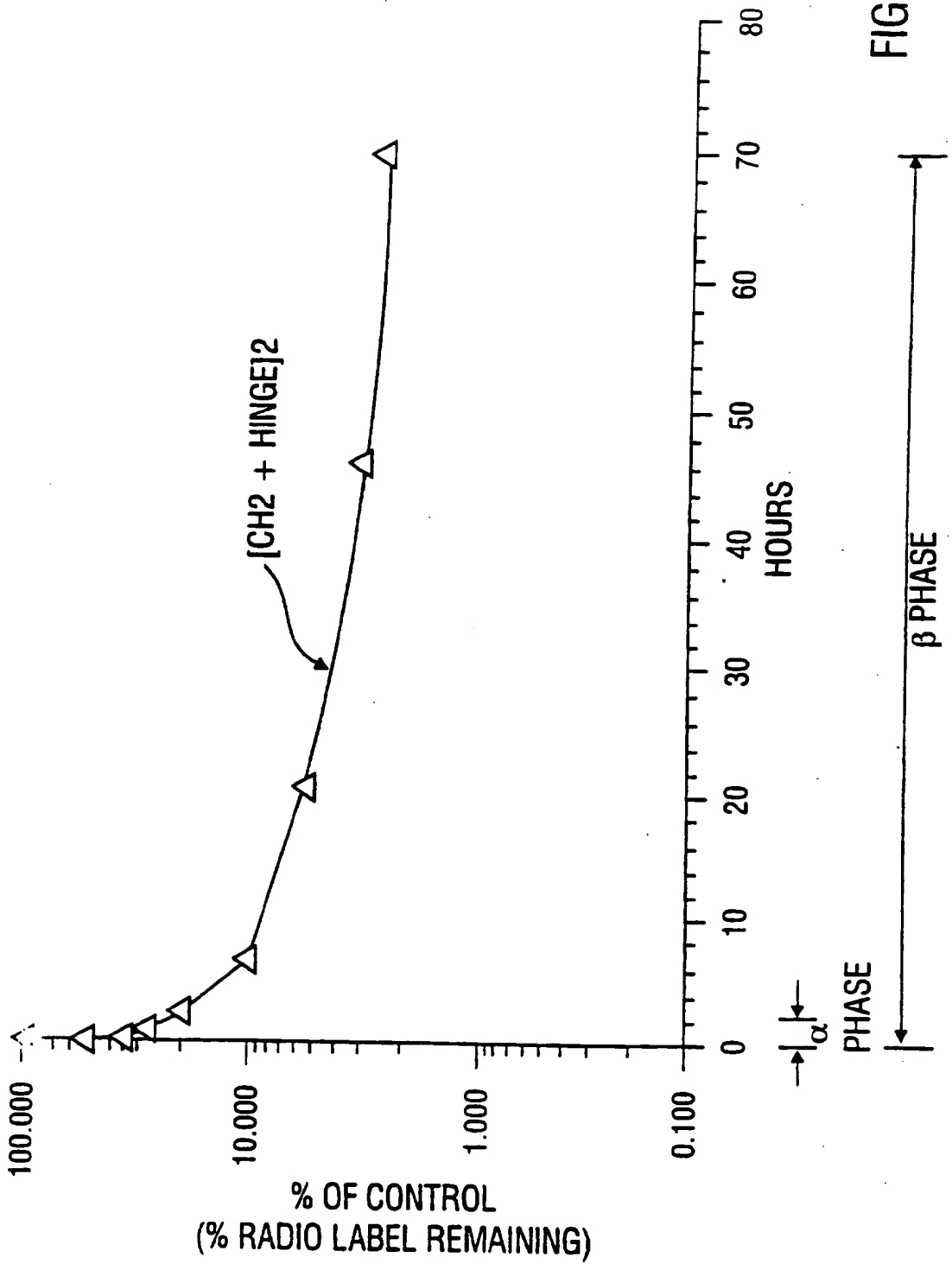
[CH2 + HINGE]2
100.000
10.000
1.000
0.100
% OF CONTROL
(% RADIO LABEL REMAINING)
0
10
20
30
40
50
60
70
80
HOURS
α
PHASE
β PHASE

FIG. 2A

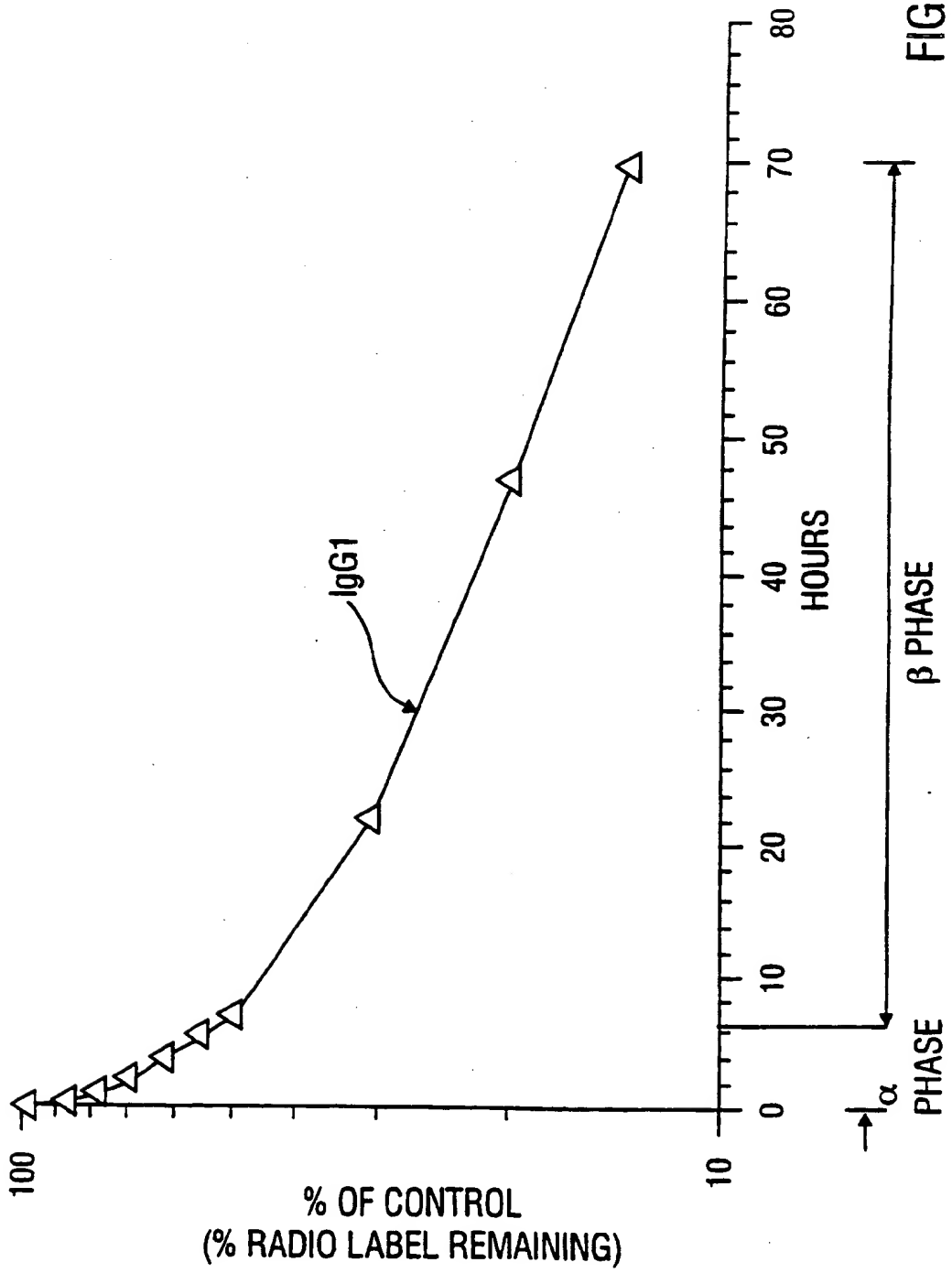
IgG1
100
10
% OF CONTROL
(% RADIO LABEL REMAINING)
0
10
20
30
40
50
60
70
80
HOURS
α PHASE
β PHASE

FIG. 2B

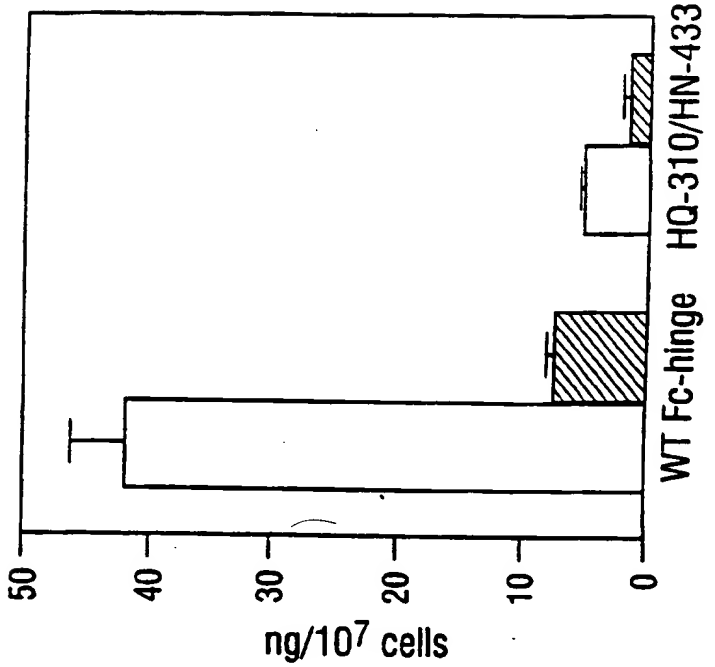
WT Fc-hinge
HQ-310/HN-433
0
10
20
30
40
50
ng/10^7 cells

FIG. 3B

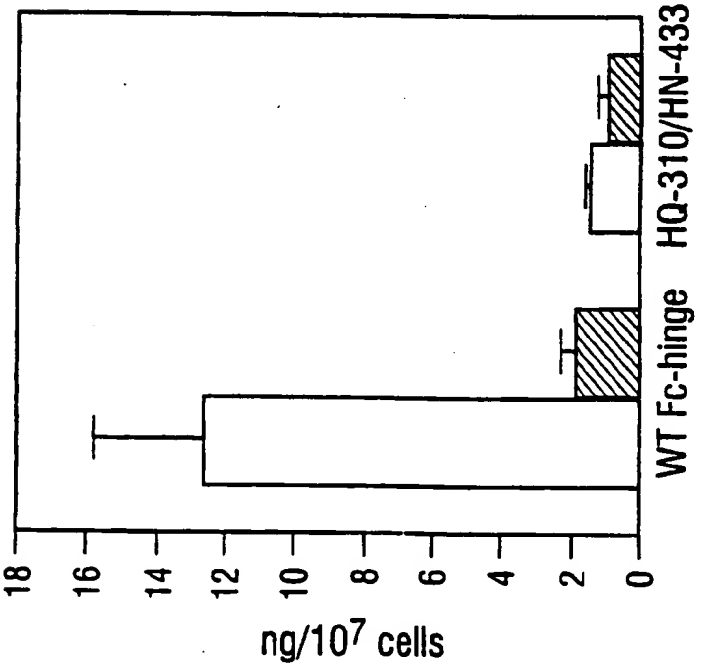
WT Fc-hinge
HQ-310/HN-433
0
2
4
6
8
10
12
14
16
18
ng/10^7 cells

FIG. 3A

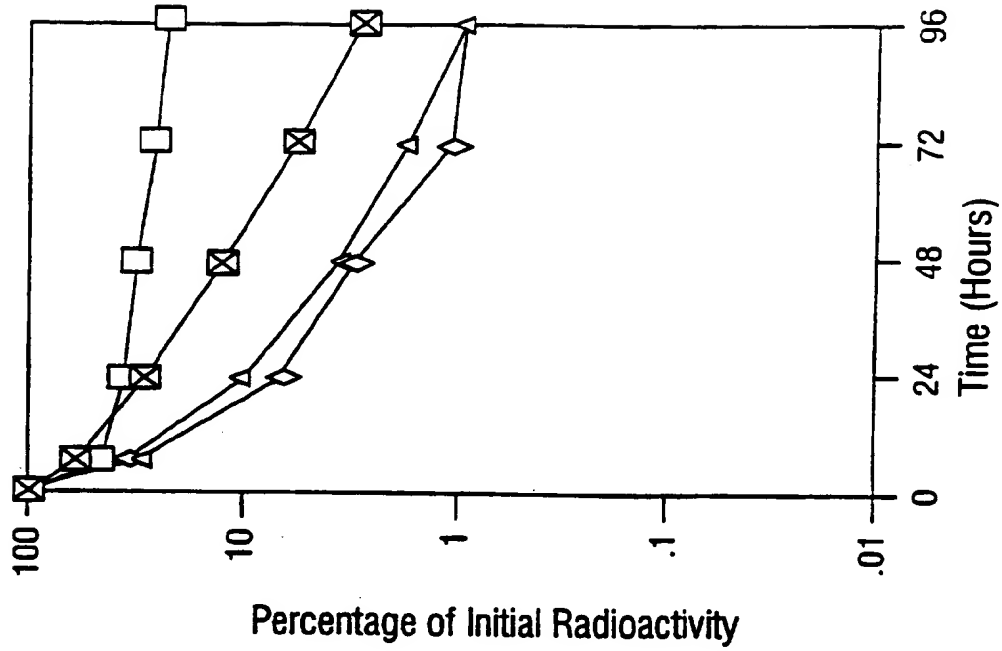
100
10
1
.1
.01
Percentage of Initial Radioactivity
0
24
48
72
96
Time (Hours)

FIG. 4B

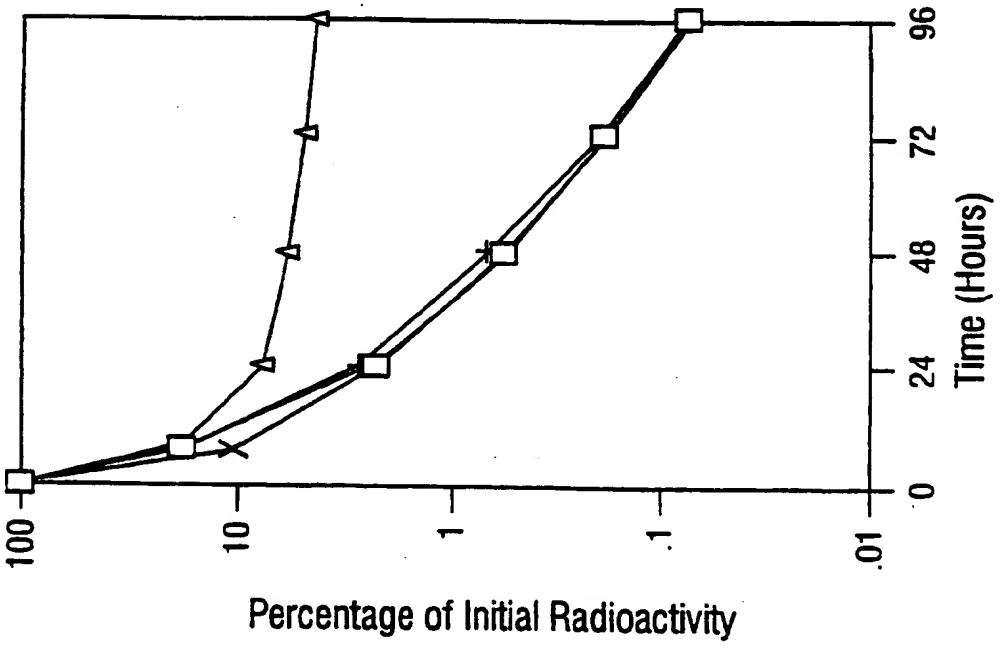
100
10
1
.1
.01
Percentage of Initial Radioactivity
0
24
48
72
96
Time (Hours)

FIG. 4A

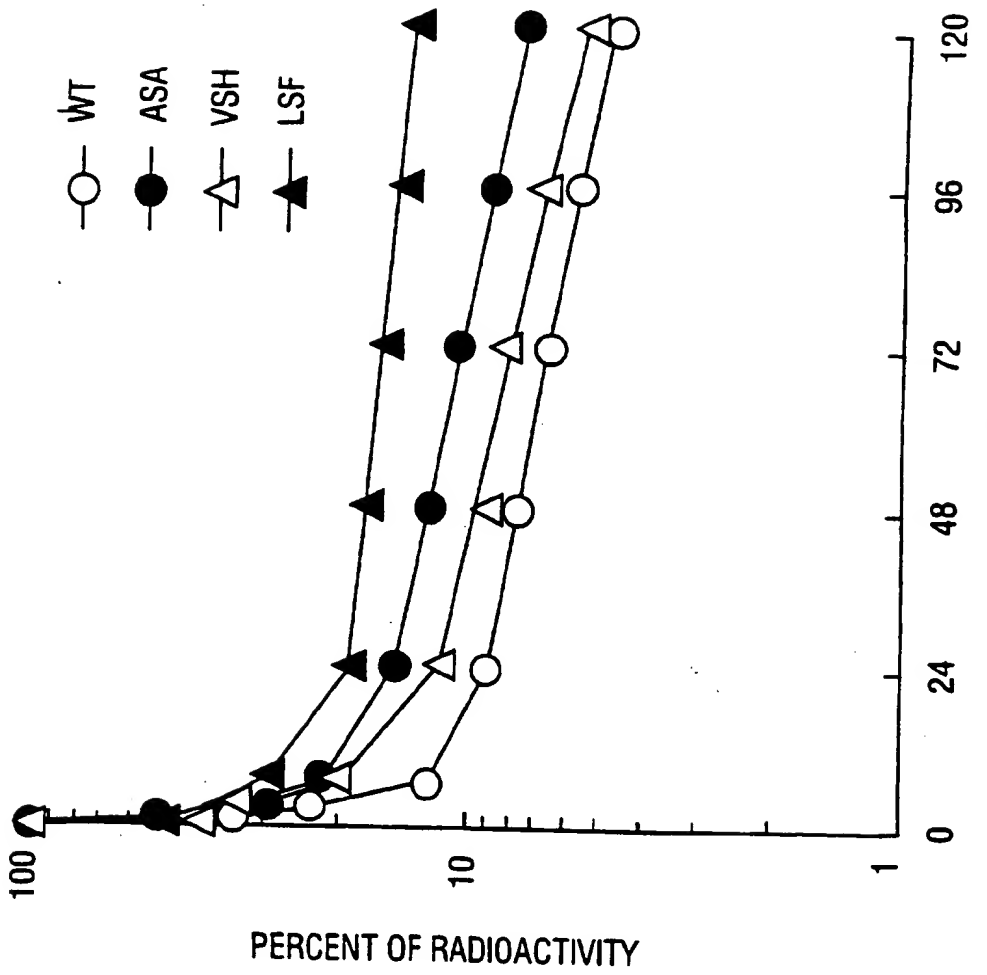
WT
ASA
VSH
LSF
100
10
1
0
24
48
72
96
120
HOURS
PERCENT OF RADIOACTIVITY

FIG. 5

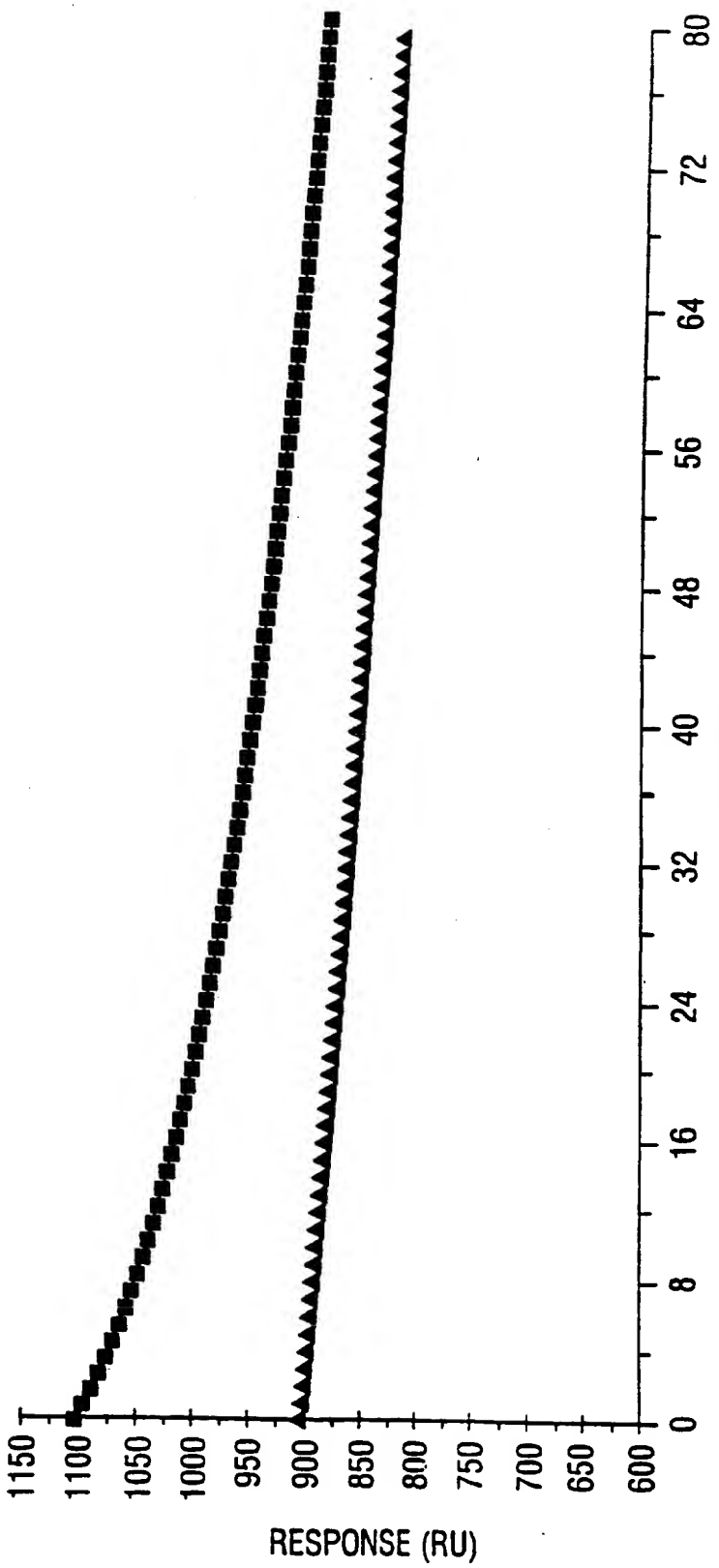
RESPONSE (RU)
1150
1100
1050
1000
950
900
850
800
750
700
650
600
TIME (SEC)
0
8
16
24
32
40
48
56
64
72
80

FIG. 6A

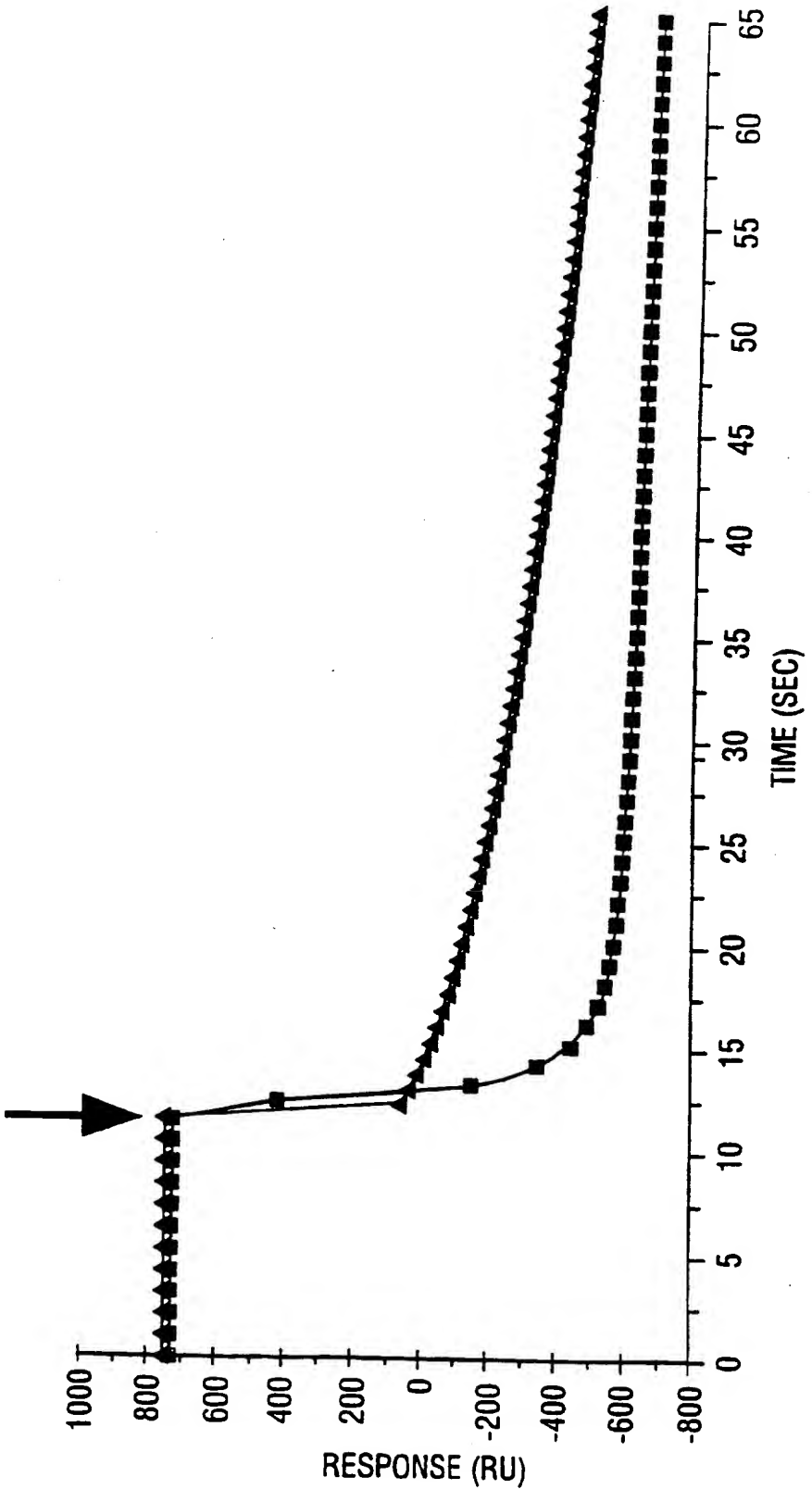
1000
800
600
400
200
0
-200
-400
-600
-800
RESPONSE (RU)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
TIME (SEC)

FIG. 6B

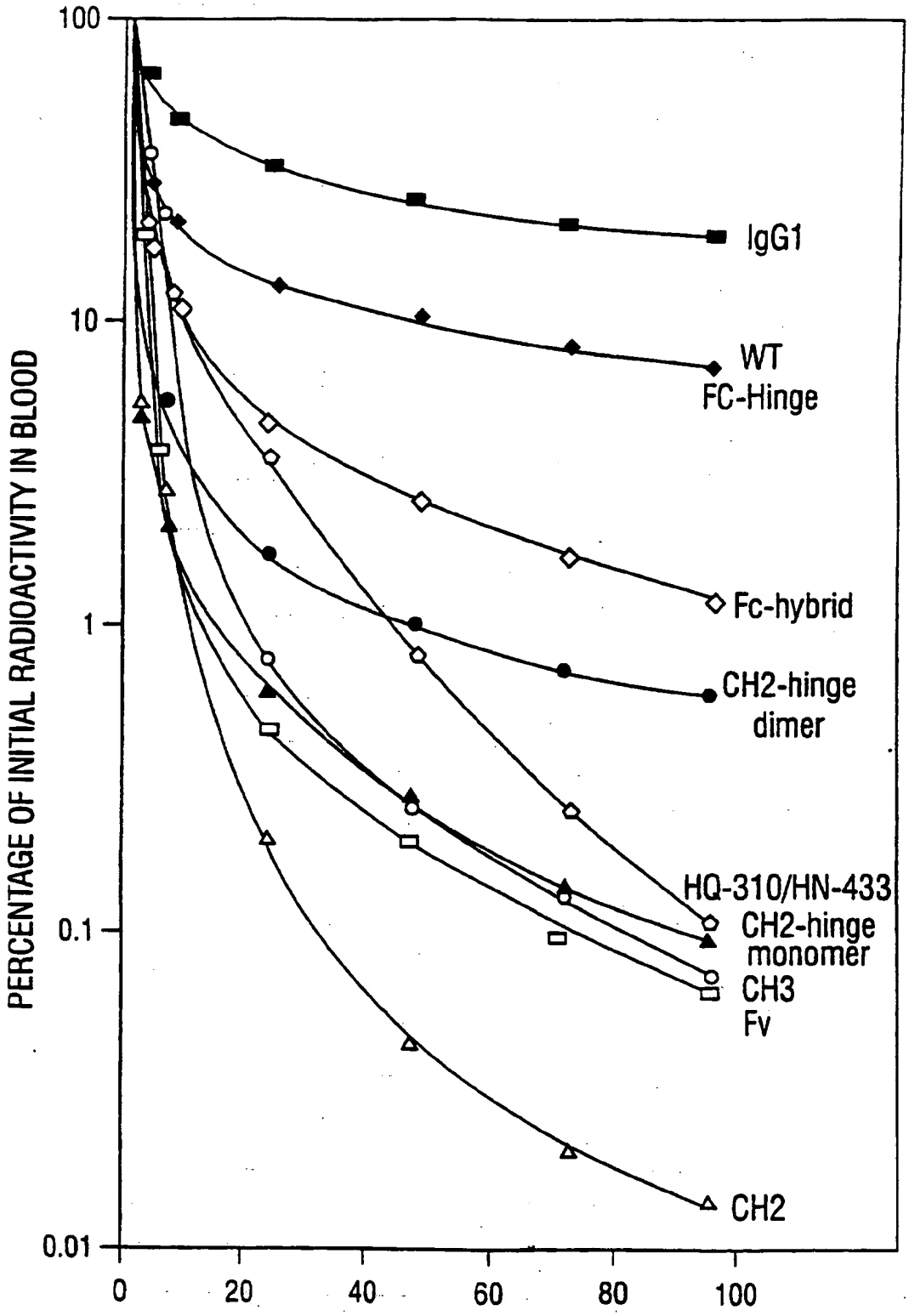
100
10
1
0.1
0.01
0
20
40
60
80
100
PERCENTAGE OF INITIAL RADIOACTIVITY IN BLOOD
IgG1
WT
FC-Hinge
Fc-hybrid
CH2-hinge
dimer
HQ-310/HN-433
CH2-hinge
monomer
CH3
Fv
CH2

FIG. 7

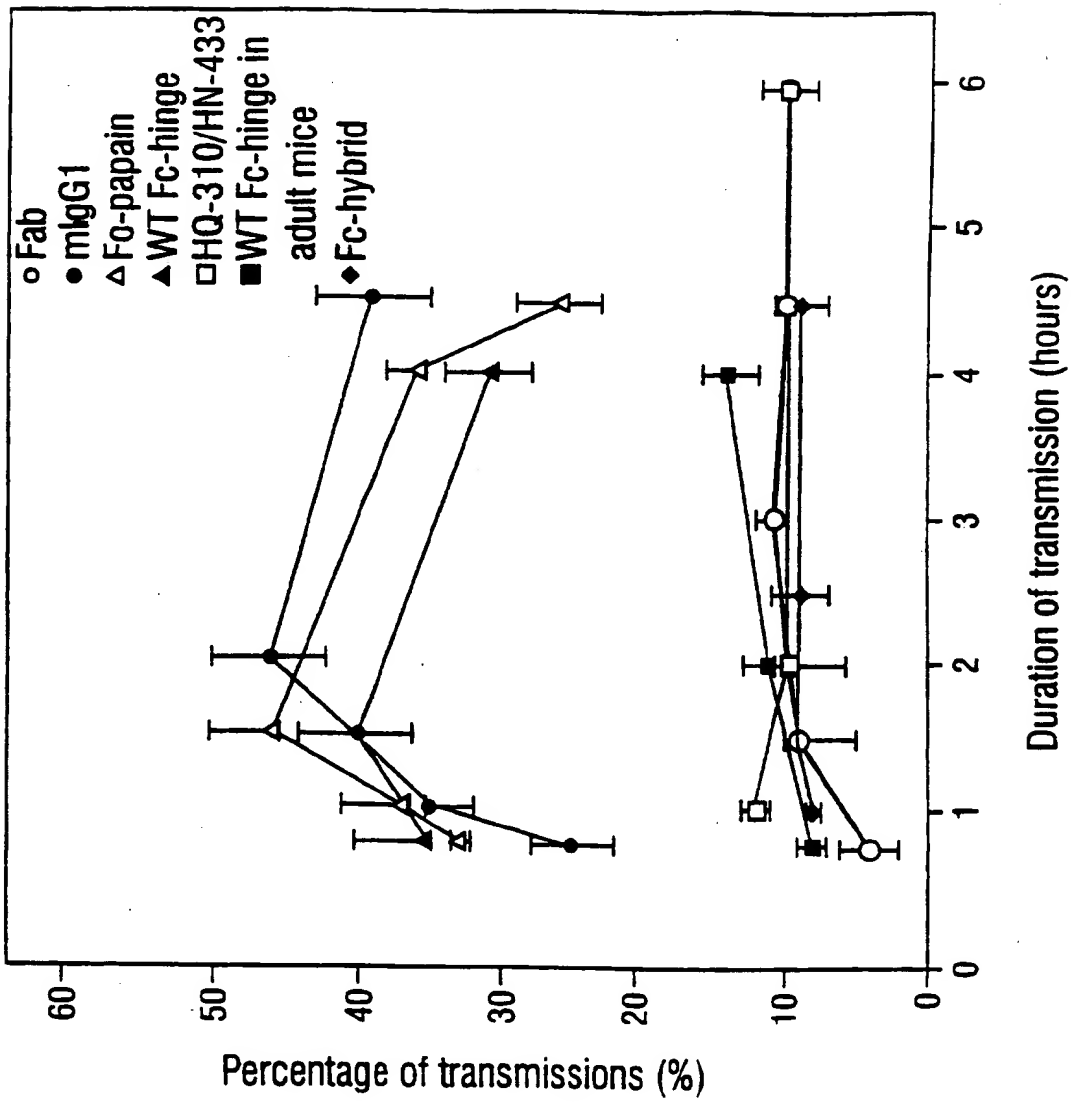
Fab
mIgG1
Fo-papain
WT Fc-hinge
HQ-310/HN-433
WT Fc-hinge in adult mice
Fc-hybrid
Percentage of transmissions (%)
Duration of transmission (hours)
0
10
20
30
40
50
60
1
2
3
4
5
6

FIG. 8

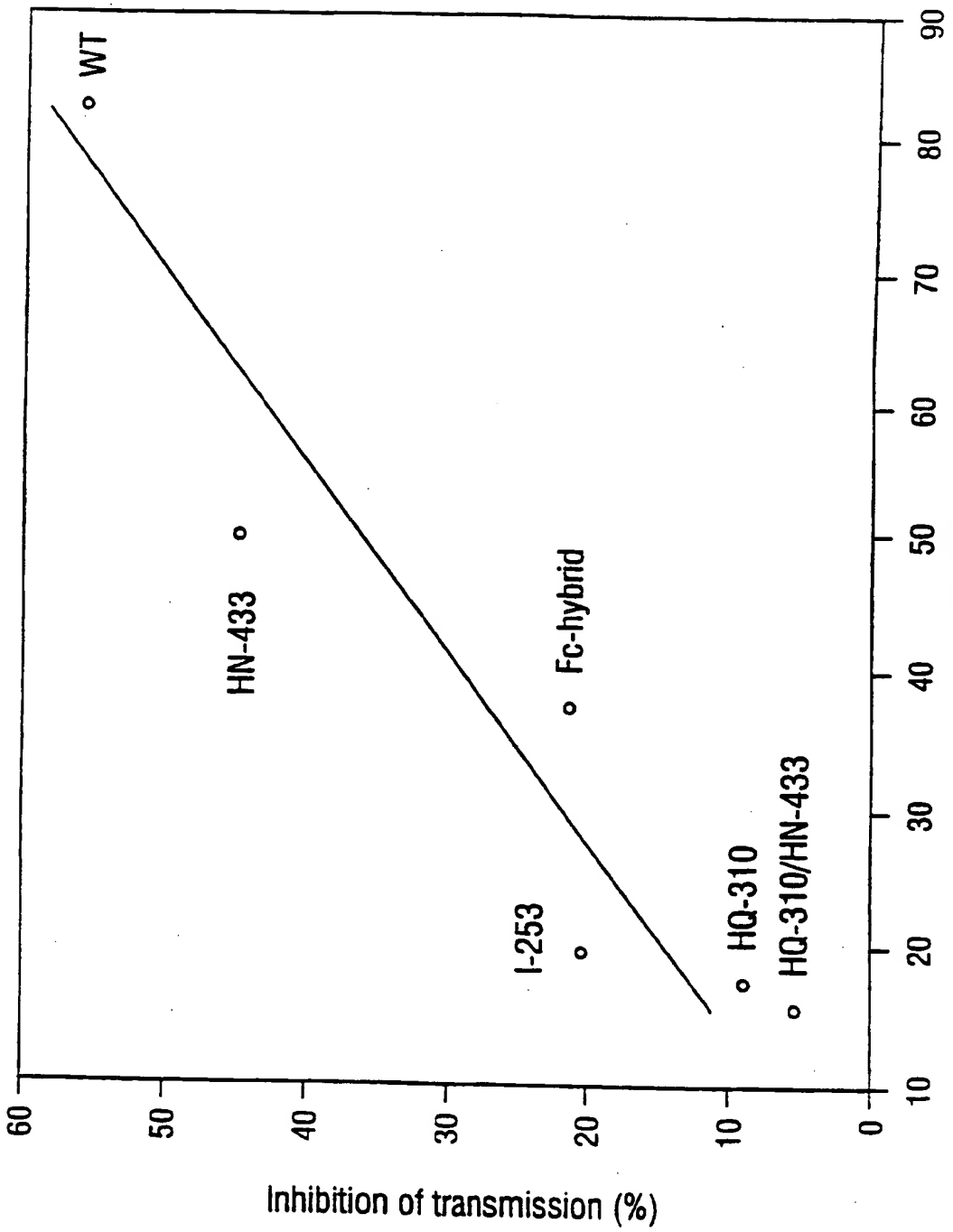
WT
HN-433
Fc-hybrid
I-253
HQ-310
HQ-310/HN-433
Inhibition of transmission (%)
Half life (hr)
0
10
20
30
40
50
60
10
20
30
40
50
60
70
80
90

FIG. 9

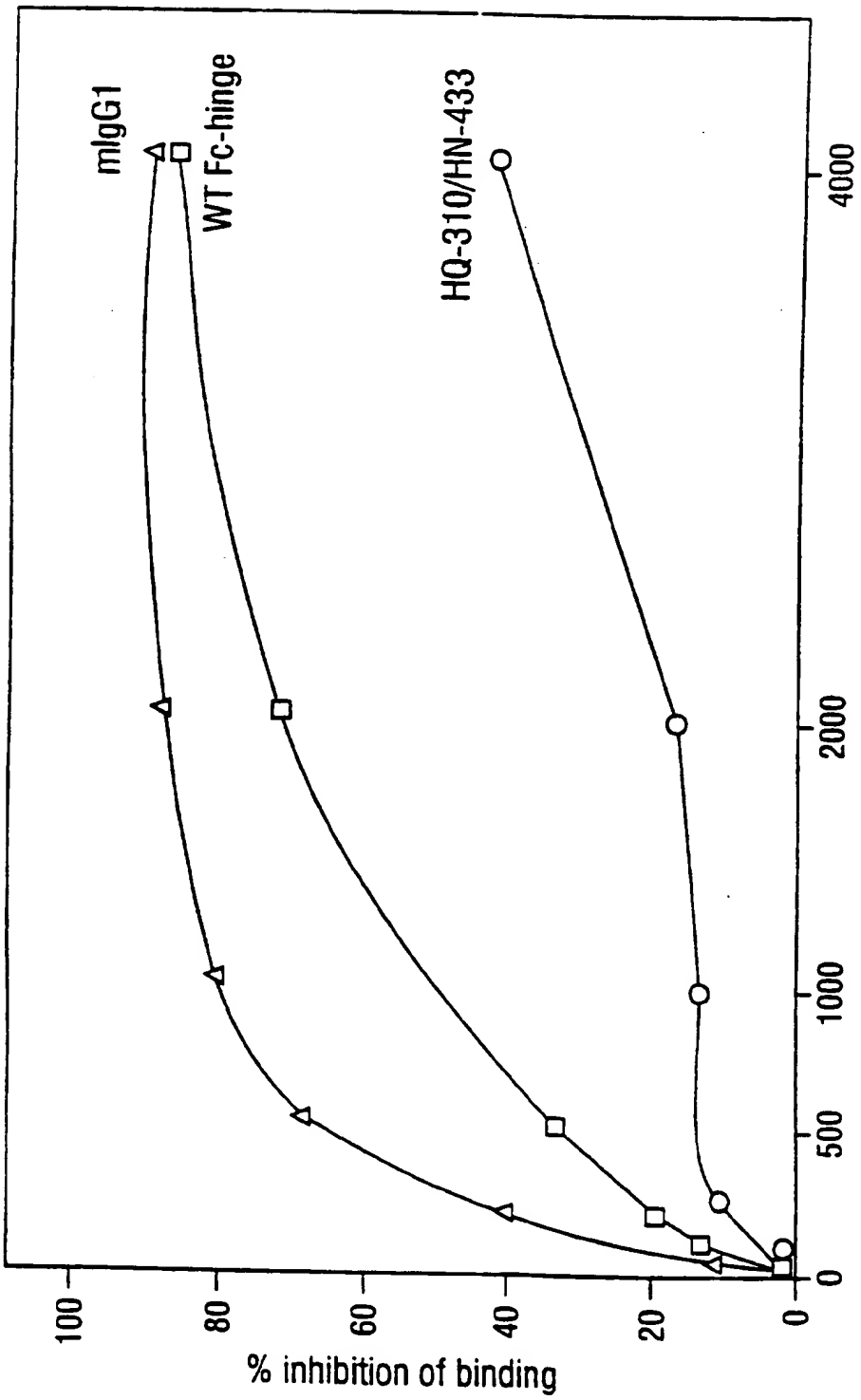
mIgG1
WT Fc-hinge
HQ-310/HN-433
% inhibition of binding
Cold inhibitor/125I-IgG1 molar ratio
0
20
40
60
80
100
0
500
1000
2000
4000

FIG. 10

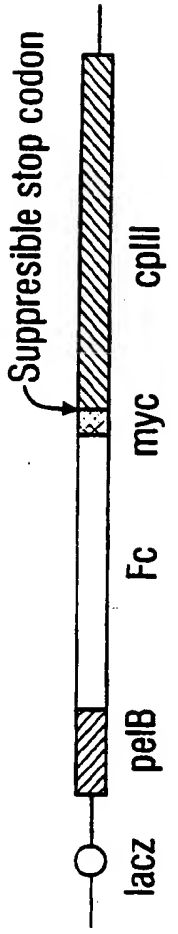

FIG. 11

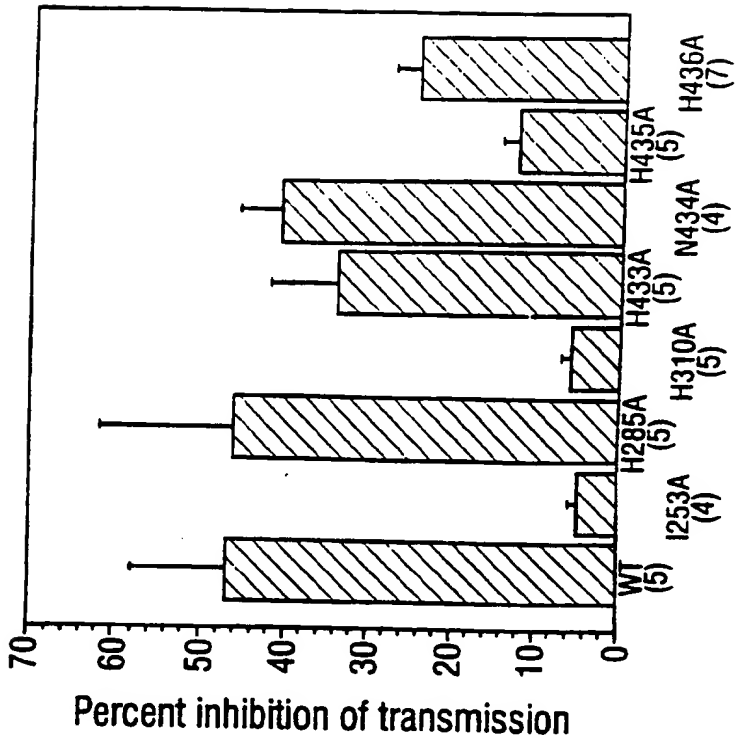

FIG. 12B

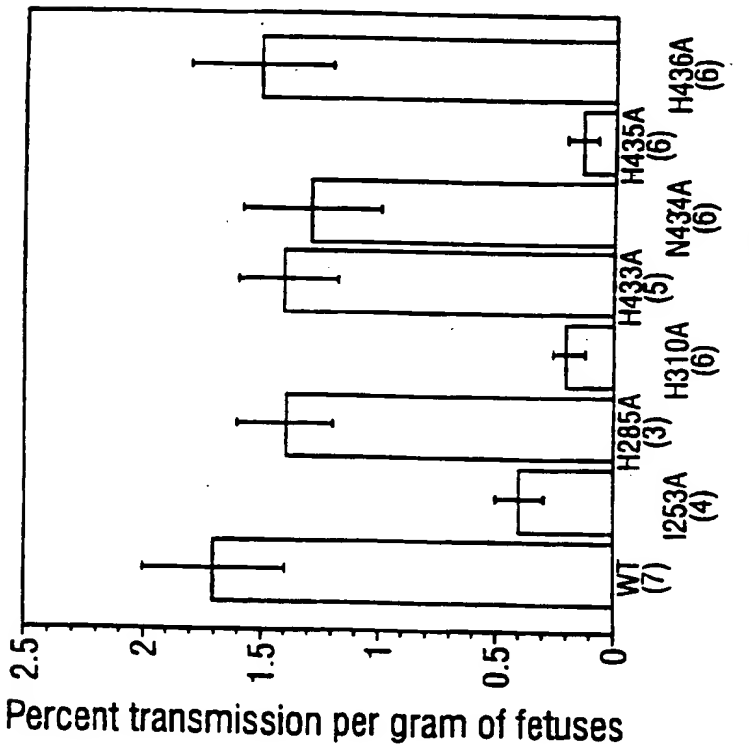

FIG. 12A

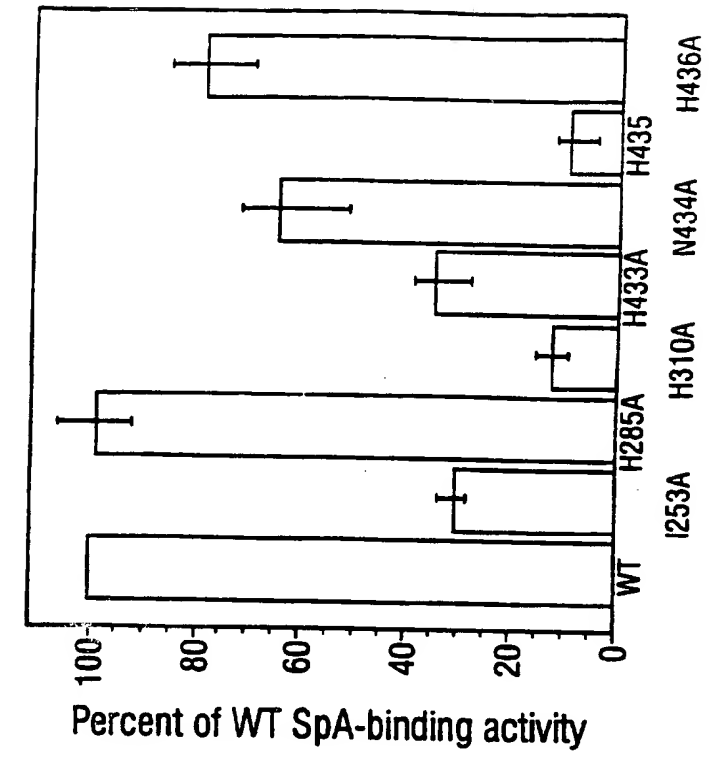

FIG. 13B

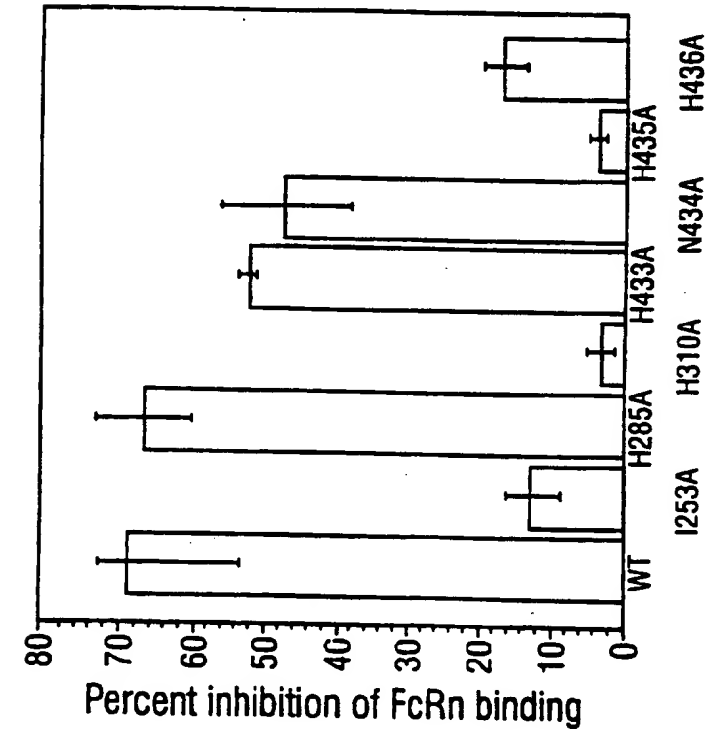

FIG. 13A